Executive Summary

1. **Project title**: Bedding strategies that promote udder health and milk quality by fostering a beneficial microbiome on organic dairy farms

2. **Project type**: Regional Proposal, New Application

3. **The legislatively-defined goals** being addressed with an estimate of the percentage of effort/funds dedicated to each are:

   (1) Facilitating the development and improvement of organic agriculture production, breeding, and processing methods.
   i. Percentage of Effort: 30%
   ii. Percentage of Funds: 30%

   This project will capture current organic industry practices of bedding management and housing facilities on organic farms, and empirically evaluate relationships between bedding management practices, bedding and udder microbiomes and their impact on animal health, milk quality and milk yield.

   (2) Evaluating the potential economic benefits of organic agricultural production and methods to producers, processors, and rural communities.
   i. Percentage of Effort: 10%
   ii. Percentage of Funds: 10%

   This project will provide a valuable budget tool to aid organic dairy farmers in evaluating the economic benefits of alternative bedding management practices, and has potential broader impacts extending beyond animal welfare and economics, to address an environmentally friendly method of managing manure as a solid rather than as a liquid.

   (6) Conducting advanced on-farm research and development that emphasizes observation of, experimentation with, and innovation for working organic farms, including research relating to production, marketing, food safety, socioeconomic conditions, and farm business management.
   i. Percentage of Effort: 60%
   ii. Percentage of Funds: 60%

   We will conduct advanced research on certified organic dairy farms to collect empirical data to define the ecological relationships of bedding, teat skin, and mammary microbiota and assess their functionality related to cow exposures to mastitis pathogens. This study is potentially transformative in establishment of new tools for risk assessment and disease prevention that are based on the integration of culture-dependent and culture-independent approaches to mastitis epidemiology.

4. The approximate distribution of percentage of effort between research, education and extension are:

A. Research: 50%
B. Education: 10%
C. Extension: 40%

5. Program Staff and their role

Project Directors:	Responsibilities and Roles
Dr. John Barlow, Ph.D. DMV, Assoc. Professor Animal and Veterinary Sciences University of Vermont John.barlow@uvm.edu	Co-coordinate the overall project with Dr. Neher, and for animal health and welfare, collecting teat and milk samples, culture-based assays of mastitis pathogens and milk quality
Dr. Deborah Neher, Ph.D., Professor (co-PD) Dept. Plant and Soil Science University of Vermont deborah.neher@uvm.edu	Co-coordinate the overall project with Dr. Barlow, and will lead the bedded pack sampling, DNA extraction and sequencing assays, and colony inhibition tests
Key personnel	
Juan Alvez, Ph.D., Outreach Professional Center for Sustainable Agriculture University of Vermont Extension juan.alvez@uvm.edu	Presentation of results at conferences and workshops. Factsheets and short articles. Update existing management practice database.
Jennifer Colby, M.S. Pasture Program Coordinator Center for Sustainable Agriculture University of Vermont jcolby@uvm.edu	Press releases, updating existing online bedded pack information resources, incorporate into farmer trainings
Consultants and advisors	
Pamela R. F. Adkins, DVM, PhD, DACVIM Food Animal Medicine & Surgery University of Missouri - Columbia adkinsp@missouri.edu	Assessment of sample collection and sample processing methods; Species identification of the isolates we collect using MALDI-ToF
Annie Claghorn foxclag@gmavt.net	30 years as commercial organic dairy producer using tie stall housing and maintaining excellent milk quality

John Cleary Organic Valley Cooperative john.cleary@organicvalley.coop	Advice and feedback (industry perspective and connection to cooperative members); resource for delivering survey materials, identifying cooperator farms, delivering outreach materials.
to be identified	Commercial organic dairy producer using free stall
Noah Fierer, Ph.D., Associate Professor Environmental Microbiology University of Colorado - Boulder Noah.Fierer@Colorado.EDU	Sample preparation and sequencing of marker genes and shotgun sequencing, and assist with interpretation of gene assembly
Sarah Flack, M.S. Organic Dairy Consultant sarahflackconsulting@gmail.com www.sarahflackconsulting.com	Translate research tools to farm practices
Sandra Godden, Ph.D., Professor Dairy Population Medicine University of Minnesota godde002@umn.edu	Assessment of the experimental design and data interpretation for the bedding management, animal health and welfare aspects of the project
Rachel Gilker, Ph.D. Editor & Publisher, *On Pasture* 64 Maple Avenue Voorheesville, NY 12186 rachel@onpasture.com	Disseminate results regionally and nationally through On Pasture publication, https://onpasture.com
Brian Jerose, President Agrilab Technologies LLC Enosburg Falls, VT brian@agrilabtech.com	Perform an economic analysis of bedding strategies, develop budget tool
Tyler Webb, M.S. stonypondfarm@surfglobal.net	11 years as commercial organic dairy producer using bedded pack

6. Critical stakeholder needs

This integrated study will identify economically sustainable bedding management strategies that reduce mastitis risk and promote udder health and milk quality. It is designed to enable the construction of management guidelines to reduce risk of mastitis, improve milk quality, and educate stakeholders about the microbiological risks and benefits of common winter housing strategies. Critical stakeholder needs are summarized in the introduction and background on pages 1 and 3, respectively, and are detailed in the rationale and significance section on page 9.

7. Outreach Plan

In collaboration with the stakeholder advisory panel, we will communicate relevant data and feasible management practices that producers can implement on their farms. To achieve this, we have created a wide-reaching outreach plan that disseminates both real-time results during each objective of the study and an integrated set of management guidelines at the conclusion of the study. Information will be published online through *On Pasture*, in extension bulletins, at regional conferences, in scientific journals, and at on-farm events. *On Pasture* is an online magazine for grass-based farmers and ranchers. It now has 100,000 readers each month, and is the most widely viewed online agricultural news source worldwide (see advisor R. Gilker letter). Outreach plans are described on pages 18 to 19.

8. Potential economic, social and other benefits

Our work addresses a crucial need to establish effective methods for prevention of mastitis on organic dairy farms. Reducing mastitis presents an opportunity for increased profitability for producers, healthier animals, improved animal welfare, and a better product for consumers. In collaboration with our advisory panel, our program will provide an economic analysis and generate a budget tool for bedding strategies detailed in the management guidelines. Dairy producers are always under pressure to maximize profitability, improve market access and meet consumer demands. Furthermore, consumers are increasingly expressing interest and concern for animal welfare and animal housing. This project will identify winter housing alternatives that enhance economic profitability through improved animal health and productivity while identifying housing alternatives that meet high-welfare standards. Economic aspects of the outreach plan are described in the background section of the introduction on page 3 and specific activities related to development of a management practice guideline to support farmer's economic decision-making are described on page 17.

9. Stakeholder Engagement

The research and survey portions of the study will be conducted entirely on commercial certified organic dairies, including a survey (mail and online option) of 200 organic dairy farmers, on-farm interviews of 40 organic dairy farmers, 6-month winter prospective repeated sampling on 20 certified organic farms, and presentation of results and best practices recommendations at farmer conferences. An important priority of the proposed study is the creation of a diverse stakeholder advisory panel of organic producers to ensure a close relationship and open communication with the wider community of stakeholders throughout the program. The group will include regional organic industry representatives, outreach specialists, economic consultants, national scientific researchers, and an organic dairy cooperative representative. Full descriptions of stakeholder engagement can be found on pages 2 and in the outreach activities section pages 18 to 19.

Bedding strategies that promote udder health and milk quality by fostering a beneficial microbiome on organic dairy farms

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Introduction

The specific **long-term goals** of this research project are to identify economically sustainable bedding management strategies that reduce mastitis risk and promote udder health and milk quality on organic dairy farms, and to develop best management practice recommendations addressing udder health microbiological risks and benefits of common winter housing strategies, with a focus on "bedded-pack" loose-housing systems. To address these specific goals, we propose a project led by a team of research and extension specialists with expertise in: 1) mastitis and milk quality (Barlow); 2) biological indicators for environmental monitoring of agricultural systems (Neher); and, 3) outreach and extension for pasture-based farms integrating bedded-pack housing systems (Colby and Alvez).

Mastitis control is identified as a **critical need** among organic dairy producers in the northeastern United States (US). Farmers and their advisors seeking information on best practices find limited science-based research conducted on organic dairy farms and must make inferences from research conducted on conventional dairy farms. The housing environment and bedding management are key factors influencing cow comfort, cow cleanliness, mastitis incidence and prevalence, and milk quality (Hogan et al. 1989, Zdanowicz et al. 2004, Bewley et al. 2017). **There is a gap in knowledge on the effect of bedding management on mastitis risk and milk quality on organic dairy farms**. **This integrated research and extension project will address this knowledge gap by identifying and communicating best practices for bedding management and mastitis prevention on organic dairy farms**. While all dairy producers rely on best management practices to support cow health and control mastitis, health management and mastitis control practices on US organic dairy farms are constrained and prevention is codified (Karreman 2009). For example, without the ability to use antibiotic medications and with few effective mastitis treatments available for use on organic farms, established mastitis control programs are modified for use organic dairy farms (Tikofsky 2010). These have resulted in some success, and there is need for continued improvement so that the organic dairy industry may be recognized as a leader in quality milk production.

There is variability of mastitis risk and milk quality among organic herds (Ruegg 2009, Cicconi-Hogan et al. 2013a, 2013b). When comparing the common housing systems, (tie-stall barns, free-stalls or bedded or composted packs), cows housed in loose housing systems appear to have improved milk quality, yet significant variation remains (Cicconi-Hogan et al. 2013a, 2013b). Compounding this uncertainty are several permutations of loose housing bedded pack management systems that create dissimilar housing environments all known as "bedded pack" or "composted bedded pack." Loose housing bedded pack systems are described as an economical alternative to conventional tie-stall and free-stall housing systems, and there is some evidence that cows have reduced rates of mastitis and improved milk quality when managed under these systems (Barberg et al. 2007, Astiz et al. 2014).

This regional integrated research and extension project will focus on addressing the uncertainty regarding mastitis risk in winter housing of organic dairy cattle and specifically develop empirical data on udder health microbiological risks and benefits to inform best management practices and economic decision making for use of bedded pack loose-housing systems. Economic decision tools that incorporate cost benefits of mastitis risk will be created. Outreach efforts will utilize multiple communication media and target multiple stakeholder communities, including organic dairy farmers, dairy farm advisors, extension specialists, veterinarians, and dairy cattle health and mastitis researchers.

Research Objectives are three-fold:

1. Assess current bedding management practices, mastitis management, animal health and milk quality on northeastern organic dairy farms.
2. Improve our understanding of associations between mastitis epidemiology, bedding microbiology and characteristics, and bedding management practices on organic farms.
3. Characterize milk, teat skin and bedding microbial community function and how it may influence mastitis pathogen prevalence.

Outreach Actions are four-fold:

1. Facilitate communication between researchers and stakeholders to ensure relevance and engagement.
2. Construct management guidelines outlining practices that reduce risk of mastitis and increase milk quality.
3. Develop a partial budget tool for annual construction and maintenance of a bedded pack incorporating animal health cost and return estimates.
4. Distribute results and tools that are applicable to the regional target area and beyond.

Stakeholder engagement

This proposal addresses the critical need to establish effective methods for mastitis prevention on organic dairy farms. Results of a prior regional survey of northeastern organic dairy farmers support the development of a research and extension project targeting mastitis control (Pereira et al. 2013). Emerging metagenomics technologies present an exciting opportunity to understand the utility of a microbiome that reduces the risk of infection for a herd. Preliminary conversations with stakeholders suggest a high level of interest and belief in the relevance of this opportunity. Continued stakeholder engagement is critical for the success of the project. Our aim is to receive continuous feedback and participation from stakeholders through the duration of the project. We will do this by establishing a **stakeholder advisory panel** that engages organic dairy farmers. Stakeholders will advise on each objective of the study prior to its implementation to ensure industry relevance and identify farm related factors that may not be obvious to researchers. We created an advisory panel comprised of commercial organic farmers that use the range of bedding strategies, regional dairy industry representatives, outreach specialists, economic consultants, national scientific researchers, and an organic dairy cooperative representative (see supporting letters).

Farmers are involved at all phases of the project from advising each objective to developing best management practices that are economically viable. Research will be performed on commercial, certified organic dairy farms, providing an opportunity to obtain realistic data on organic systems and encourage creative interactions between producers and researchers. Concurrent with research, ongoing results will be disseminated via social media, internet-based publications, stakeholder advisory meetings, and conferences.

Background Information

Mastitis is a critical topic of concern for organic dairy farmers. Mastitis remains one of the most costly health concerns of dairy cattle in the US and is one of the top priority diseases identified by the 2015 Agriculture Research Service stakeholder survey, and in the 2016 USDA Animal Health Dairy stakeholder webinar series (Julie M. Smith, personal communication). Organic dairy farmers in the northeastern US identify mastitis as a top animal health challenge area and mastitis control as a key research priority (Pereira et al. 2013).

Mastitis is defined as inflammation of the mammary gland and is primarily due to bacterial infections, yet can be caused by many different species of bacteria, yeast and fungi (Watts 1988). Mastitis associated economic losses are due to lost or reduced milk production, increased veterinary and treatment costs, increased labor, low product quality resulting in low premiums, and animal replacement costs due to culling (Bar et al. 2008). Even after recovery, the udder of a cow may not return to optimal functional capacity (Akers & Nickerson 2011), resulting in prolonged production losses. On average, a case of clinical and subclinical mastitis costs USD$ 367/case-year and USD$ 130/case-year, respectively (Halasa et al. 2007). Although recent national data is lacking, estimates from the 1990s showed that mastitis losses were between USD$ 1.5-2.0 billion per year in the US (Middleton et al. 2014). In a study of 20 organic dairy herds in Wisconsin, an average of 20% of cows experienced a case of clinical mastitis and 9% of cows were culled for mastitis per year (Pol & Ruegg 2007). Other studies identify similar rates of clinical mastitis among organic dairy herds (Sato et al. 2005, Ruegg 2009, Richert et al. 2013, Levison et al. 2016). Generally, clinical mastitis cases are the "tip of the iceberg" and subclinical infections dominate on most dairy farms. Chronic subclinical mastitis is often under-recognized by many farmers (Barlow et al 2009). Using conservative cost estimates an 60 cow organic dairy farm would have USD$ 6000 in annual costs attributed to mastitis. Reducing the incidence of mastitis in this herd by 50% would result in a savings of USD$ 30,000 over a decade. Such a savings is significant under today's increasingly tight margins for organic milk production. There is an economic opportunity for organic dairy farmers to invest in science-based management strategies to reduce mastitis incidence and prevalence.

Needs of Organic Dairy farmers in Vermont, the northeast and the US. Vermont is ranked sixth by number of organic dairies per state and first by number of organic dairies per square mile in the US; Vermont includes 67% of New England dairy farms (USDA-NASS 2016). More than 200 dairies are certified currently by Vermont Organic Farmers, LLC, accounting for approximately 25% of Vermont's dairy farms, 12% of adult dairy cows, and 5% of the milk production (O'Hara & Parsons 2013, VAAFM 2017). Serving Vermont's dairy industry, including organic dairy producers, is a key component of research and extension efforts at the University of Vermont (UVM). Herd size, types of facilities, milk production levels, and mastitis control practices of Vermont organic dairy farms are similar to those of other northern tier US states that lead in organic dairy production (e.g., Wisconsin, New York, Pennsylvania, Maine and Minnesota) (O'Hara & Parsons 2013, Stiglbauer et al. 2013, J. Barlow unpublished). With 200 dairy farms readily accessible to UVM, Vermont is an excellent "living laboratory" to conduct research of regional and national relevance to organic dairy farming.

A number of recent research publications demonstrated the importance of mastitis control on US organic dairy farms (Sato et al. 2005, Pol & Ruegg 2007, Pereira et al. 2013). In an OREI-funded survey of 163 organic dairy farmers from the Northeast (New Hampshire, New York, Pennsylvania, Maine, Massachusetts, and Vermont), 81.5% of respondent farmers described mastitis control as a challenge, placing mastitis as a major health concern (Pereira et al. 2013). In a survey of 183 organic dairy herds from three states (New York, Oregon, and Wisconsin), rates of farmer observed clinical mastitis ranged from 0 to 1.3 (mean 0.3) cases per 305 cow days (equivalent to 35 clinical cases per 100 cows per year) (Rickert et al. 2013). The geometric mean bulk tank milk (**BTM**) somatic cell count (**SCC**) of the organic herds in this study population was 195,000 (range 45,000 to 724,000) cells per ml (Cicconi-Hogan et al. 2013a). SCC is a measure of milk quality related to mastitis prevalence, and BTM herd-level SCC levels above 200,000 cells per ml indicate an opportunity for mastitis control (Schukken et al. 2003). In this past multi-regional study, increased BTM SCC was associated with smaller herds, presence of *Staphylococcus aureus* in the BTM, and milking procedures (Cicconi-Hogan et al. 2013b). Increased rates of farmer-identified clinical mastitis were associated with smaller herd size, absence of grazing, and presence of tie-stall or group housing compared to pasture or free-stall housing (Rickert et al. 2013). However, the association between rates of clinical mastitis and the types of bedding used or the bedding characteristics were absent, emphasizing the current gap in knowledge.

Impact of bedding management on mastitis risk in dairy herds. To the best of our knowledge, no prior studies have specifically evaluated the effect of different housing and bedding management systems on risk of mastitis in US organic dairy herds.

The environment, and especially bedding, is an important source of bacteria and fungi that can be transmitted to the teat and mammary gland (Hogan & Smith 1997, Zdanowicz et al. 2004). For many opportunistic pathogens, including *Escherichia coli* and the "environmental streptococci" the source of new intramammary infections is the environment, especially fecal contamination of the cow's environment. These "environmental" organisms are differentiated from the contagious mastitis pathogens, for example *Staphylococcus aureus*, where the source of new infections is due to spread from infected cows in the herd and milking procedures play an important role in transmission. Thus, bedding and environmental management is but one important component of an integrated mastitis control program (Tikofsky 2010). Different bedding materials and systems appear to influence the risk of mastitis in dairy herds (Goldberg et al. 1992, Hogan et al. 1989). The traditional theory is that some bedding materials and systems have smaller numbers of specific opportunistic pathogens and, thus, reduce the level of exposure of the mammary gland to these organisms. For example, inorganic bedding materials, such as sand, generally have lower bacterial loads compared to organic materials. Yet, bedded-pack systems use organic material and do not increase the prevalence of mastitis compared to sand bedded free stall systems (Eckelkamp et al. 2016b). Further, there appears to be no difference in the use of procedures for milking hygiene to control mastitis for similar sized organic and conventional farms in New York, Oregon and Wisconsin (Stiglbauer et al. 2013). An alternative explanation is that variation in bedding material and management system influence the risk of intramammary infection by affecting the teat skin microbial community (see "Impact of microbiome" below). A comparison of mastitis risk among cattle housed on different winter bedding systems, while accounting for other management practices that influence mastitis risk, including milking and udder hygiene, will help resolve these issues and guide bedding management decisions within a comprehensive mastitis control program.

Management intensive or rotational grazing herds have reduced rates of mastitis compared to confinement farms (Goldberg et al. 1992, Rickert et al. 2013), and free-stall herds have reduced rates of mastitis compared to tie-stall herds (Rickert et al. 2013). Yet, bulk tank SCC variation is not explained entirely by housing (i.e., free-stalls, tie-stalls, versus compost bedded pack) (Eckelkamp et al. 2016b). Therefore, it is a logical next step to address mastitis control by focusing on winter housing conditions for organic cows. Comparisons of mastitis rates related to factors such as bedding type and facility design must account for other factors known to influence mastitis risk, including herd size, level of milk production, and application of milking hygiene and mastitis management practices. Increased age of housing facilities (i.e. cow barns) is a factor that was associated with less-desirable parameters of BTM quality and increased rates of clinical mastitis (Cicconi-Hogan et al. 2013b, Rickert et al. 2013). Organic dairy farmers with aging facilities may be looking for sustainable and cost-effective housing options, and bedded-pack systems may be an affordable alternative that improve cow comfort, reduce lameness, integrate well in pasture-based production systems and reduce mastitis risk (Astiz et al. 2014, Barberg et al. 2007).

Bedded Pack. The future of housing systems for lactating dairy cattle is shifting toward improved cow comfort. Bedded pack loose housing systems are designed for cow comfort (Endres et al. 2007, Bewley et al. 2017). Public perceptions and animal welfare concerns are motivating elimination of tie-stall and restricted movement cattle housing systems (Barkema et al. 2015). As farmers consider new or renovated facilities, bedded-pack loose-housing systems may offer an attractive housing system that aligns with consumer preferences for the future of dairy cow housing (Fig. 1). In loose-housing bedded-pack or composted bedded-pack systems there are fewer foot and leg injuries than free-stall barns (Lobeck et al. 2011, Burgstaller et al. 2016). There are also reports of decreased incidence of mastitis for dairy cows on compost bedded pack (Barberg et al. 2007, Astiz et al. 2014). Bedding managed for low moisture and high temperature reduce counts of bacterial pathogens (*Staphylococci*, *Streptococci*, and *Bacilli* species) and improve herd hygiene (Black et al. 2013, Eckelkamp et al 2016a). Traditionally, bedded packs have been thought to increase risk of mastitis due to the presence of pathogenic bacteria (Black et al. 2014) and the favorable moisture and temperature for the growth of these pathogens (Favero et al. 2015). However, neither of these measures have been demonstrated to be consistent predictors of mastitis risk. We believe we are among the first research groups to consider the potential role of the bedding microbiome, the udder skin microbiome, and the putative milk microbiome in mastitis risk and BTM quality on organic dairy herds and in bedded-pack systems.

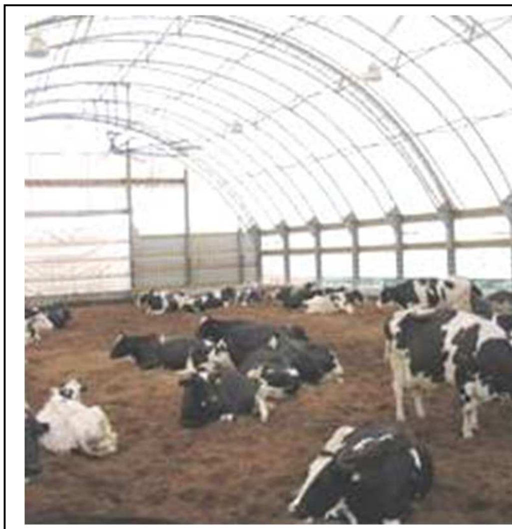


Figure 1. Bedded-pack winter housing on an organic dairy farm in Vermont (*photo credit J. Colby Vermont Pasture Program*)

Impact of the Microbiome: Genetic sequencing of the microbial community is redefining traditional understanding of the mammalian relationship with microbes, evincing a paradigm shift away from a simple host-pathogen interaction to considering infection as a manifestation of dysbiosis, or imbalance, of the mammary microbial community (Keeney et al. 2014, Rainard 2017). For example, DNA sequences of *S. aureus* and *Streptococcus uberis* are present in milk samples from cases of clinical mastitis of dairy cows but also in healthy quarters, on the skin, in the mouth and gut as part of normal microbiota (Rainard 2017). The "mastitis as dysbiosis" concept is not without controversy, especially the presence of a normal milk or intramammary microbiome (Rainard 2017). For decades, mastitis researchers have described the normal intramammary environment as sterile, with any introduction of bacteria leading to some degree of local or systemic inflammation (Bramley et al. 1992, Rainard 2017). A growing body of evidence suggests that cow teat skin, bedding and, perhaps, even the intramammary milk microbiome are composed of a diverse community of bacteria and fungi, with both positive and negative implications for susceptibility to mastitis. Culture-independent DNA-sequence-based techniques suggest that microbiomes are composed of bacterial and fungal communities refractory to identification by traditional culture-based methods (van Baarlen et al. 2013). While the source of the microbial DNA in bovine milk samples remains controversial (Rainard 2017), it is expected that the microbial community structure of either bedding or cow skin may be more complicated than can be recognize by culture-based methods alone.

Bedding is an important source of bacteria and fungi that can be transmitted to the teat and mammary gland (Halasa et al. 2009, Eckelkamp et al. 2016a), potentially changing the ability of these communities to resist dysbiosis. This potential necessitates the integrated comparison of all three habitats. Identifying differences in community structure or function in systems housing cows with lower prevalence of mastitis would lead to the identification of beneficial organisms or cohorts, and associated management practices to encourage their establishment. There is a critical need to define the ecological relationships of bedding, teat skin, and mammary microbiota and assess their association with mastitis resistance.

Factors that affect microbiota. Diet, herd management, animal housing, animal density, hygienic procedures during milking, or animal genetics and age potentially affect the microbial community (Falentin et al. 2016). High milk SCC is inversely associated with bacterial diversity (Rodrigues et al. 2017) suggesting a commensal microbial community that provides a balance or steady state that mitigates pathogen colonization or infection (Rainard 2017). Controversially, chemical teat disinfection (the cornerstone of today's mastitis control programs) has been hypothesized as deleterious to this steady state by destroying beneficial bacteria (Vacheyrou et al. 2011). Detailing the mechanisms of interaction and resource utilization of a local microbial community will identify microbial functions that resist or allow dysbiosis.

Ongoing or recently completed significant activities related to the proposed project. We conducted multiple searches of the USDA Current Research Information System (CRIS) database and identified **no duplication of efforts**. A search on the terms **"organic** and **dairy** and **mastitis"** resulted in 28 hits, and 2 were found to be potentially relevant. The completed project "Impact of organic management on dairy animal health and well-being" (WIS01351; PI P. Ruegg, "Project C.O.W") had a major component related to mastitis control. Materials generated by that project provide important background material referenced in this proposal. Specifically, project C.O.W. provides an excellent set of resources for our studies, including a validated survey instrument and housing survey tool that we propose to modify to incorporate additional parameters not captured in this previous project, and to use under Obj. 1. The survey tools administered for project C.O.W. are available at http://milkquality.wisc.edu/organic-dairies/project-c-o-w/. As described in the introduction, their prior research supports the association between housing and mastitis risk (Cicconi-Hogan et al. 2013b, Rickert et al. 2013, Stiglbauer et al. 2013). The completed project "Enhancing animal care strategies on organic dairy farms" (MIN-02-G02; PI B. Heins) was focused on parasite (e.g., pest flies) control, calf feeding and calf management on organic dairy farms, and Dr. Heins continues to focus on these areas of research (personal comm.).

A search on the terms "bedded pack and dairy" resulted in 33 hits with no duplication of efforts. A newly established Hatch project "Evaluation of the association between the bacterial communities of the mammary gland, the gastrointestinal tract, and the environment of dairy cattle" is being led by Dr. Pamela Adkins. This research team is evaluating the effect of heat stress on the fecal, teat skin, milk, and bedding microbiome and the prevalence and distribution of staphylococcal species on dairy farms using different housing environments. They will evaluate the staphylococcal populations in BTM samples, composite teat skin samples, and bedding samples from 20 dairy farms in Kentucky, including 10 compost bedded pack barns and 10 sand bedded free stall barns. While regional differences and exclusion of organic herds in Dr. Adkins' study design distinguish the projects, potential synergism has led Dr. Adkins to agree to serve as a scientific advisor on our stakeholders' panel (see Adkins letter of support).

A CRIS search on "bedding and dairy mastitis" resulted in 20 hits. Many of the same projects were identified. The NIFA funded project "Application of microbiome techniques to mastitis control" (WIS01743; PI P. Ruegg) appeared to have the greatest potential for overlap with our proposed project. Her preliminary results reported in the CRIS system supports our approach. Ruegg et al. indicate that exposure to different bedding types influences the milk microbiome of healthy cows, and the researchers describe a healthy milk microbiome. There is no evidence that her research was conducted with organic herds or on herds using bedded-pack housing, suggesting our studies will be complementary.

Outside of the USDA funding system, we are aware that Dr. Sandra Godden is leading a national study "Investigating the relationship between bedding bacterial counts, bedding characteristics and management practices with udder health on 188 U.S. dairy farms." In this study, there are 6 bedded-pack herds enrolled (S. Godden personal comm.). Dr. Godden has agreed to serve as a scientific advisor on our stakeholders' panel (see Godden letter of support). Further, we plan to use the same laboratory and bedding culture methods applied in her research to allow comparison of the two studies.

CRIS searches also identified our Hatch funded pilot study (VT-H02203) "Integrated bedded pack management and fly control reduce mastitis risk by promoting a beneficial teat skin microbiome" (PIs Barlow and Neher, end date May 2018) conducted on a single commercial organic dairy farm. Preliminary data from this pilot study are reported in this proposal.

Prior regional surveys of northeastern US organic dairy farmers. Researchers at the University of New Hampshire, led by Dr. Andre Brito, have conducted two organic dairy industry needs assessment surveys during the past decade. A summary of the results of these surveys has been published (Pereira et al. 2013). Under the USDA-NIFA funded project (# NHW-2010-01932), Dr. Brito and colleagues conducted producer focus group surveys of 35 organic dairy producers from four states (Maine, Vermont, Pennsylvania, New York), and subsequently surveyed the 1200 members of the Northeast Organic Dairy Producers Alliance, of which 183 (15%) responses were returned. Mastitis was rated the second most important “Animal nutrition, health, and reproduction” challenge, and “Controlling mastitis was a challenge or somewhat of a challenge for 81.5% of respondent farmers” (Pereira eta al. 2013). Farmers were not asked specifically to comment on the need for research on preventative practices of mastitis control practices, such as nutrition, housing or the use of vaccines or biologics. Consistent with the USDA guidelines for organic certification of dairy livestock (USDA-AMS & NOFA-VT), we propose that research on preventative practices for mastitis control including appropriate housing and hygiene is justified and needed.

Preliminary Data generated by Barlow and Neher Labs. The apparent paradox of greater pathogen counts yet less disease on bedded packs suggests that organic dairy producers may benefit from integrating bedded-pack technologies into their farm systems. On organic farms in Vermont, there is evidence of reduced bulk tank SCC and lower clinical mastitis incidence for bedded-pack herds compared to tie-stall herds (Barlow et al. unpublished; Fig. 2), supporting the hypothesis that bedded-packs are associated with improved udder health. To explore the dynamics of this relationship, our group used culture-independent methods to characterize the structure of the bacterial and fungal communities of bedding and teat skin and intramammary gland of healthy and infected quarters on an organic dairy farm utilizing bedded pack (pilot herd). Intramammary infection status was determined by milk culture (Middleton et al. 2017) and teat skin and milk microbiota of infected and uninfected udder quarters were characterized. On this farm, where all cows are exposed to the same bedded-pack, cows with and without mastitis had similar teat skin bacterial communities. In contrast to skin, bacterial community structure (though not necessarily membership) was distinct between milk samples of healthy and infected mammary glands. For example, *Staphylococcus* species had similar abundance on teats (i.e., uniform cow exposure), and were present in both healthy and infected mammary glands. However, in the infected quarters, this genus was often dominant (33.4% mean relative abundance), while in healthy quarters it was present but not dominant (17.0% mean relative abundance). One hypothesis is that the virulence of putative pathogens colonizing the healthy teat is regulated by the “beneficial” microbial community, either by direct inhibition or competition for resources, preventing an opportunistic pathogen

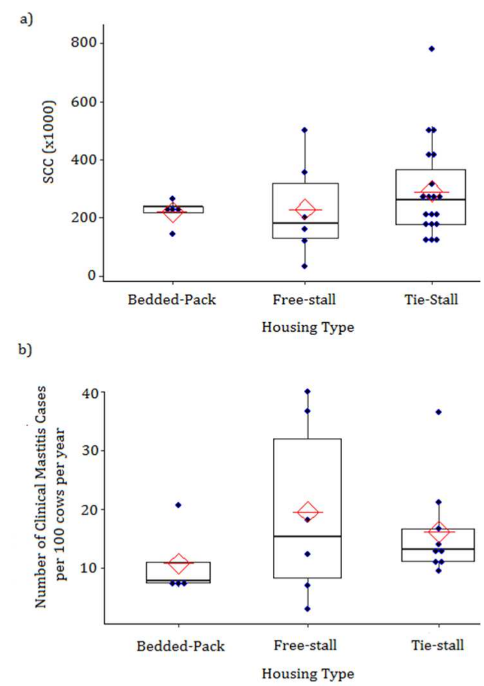


Figure 2. Comparison of milk quality and mastitis rates on organic dairy farms in Vermont. a) 3-month average bulk tank milk somatic cell count on 30 farms; b) Farmer reported annual incidence of clinical mastitis cases per 100 cows on 19 farms. Red diamonds = mean by housing type; points are values for farms demonstrating less variation for bedded-pack herds.

from establishing a quorum to trigger the production of toxins (Harrison et al. 2008, Le & Otto 2015). In some cases, bedding may be contributing both beneficial and pathogenic microbes (Table 1; Neher et al. unpublished). Fungal bedded-pack taxa associated with both healthy (*Penicillium* and *Cryptococcus*) and infected (*Wallemia, Debaryomyces, Aspergillus, Aureobasidium*) teats and mammary glands were identified on the pilot herd during July and August of 2015. The importance of these fungal taxa to udder health is not established, yet potentially beneficial taxa such *Penicillium* represented an average of 23.3% of the fungal community in healthy mammary glands while *Aspergillus* was 22% of infected mammary glands (Table 1). The combined community sequencing and culture-based approaches described in Obj. 2 will generate data to test the hypothesis that bedding strategy affects mastitis risk on organic dairy farms. Completing this objective will identify beneficial and pathogenic taxa that impact udder health. Further, the shotgun metagenomic assays we propose in Obj. 3, could be transformative in identifying the mechanism by which these taxa functionally interact to resist dysbiosis and prevent infection.

In a second pilot study of five bedded-pack farms, management technique affected the microbial community structure of bedding. In general, the microbiome of the three farms that used a deep, warm, static pack built from mulch hay and wood chips were more similar to each other than the sawdust tilled pack and the mesophilic static pack (Fig. 3). These preliminary data suggest that farmers can use current bedding management tools to control the microbiota to which their cows are exposed. Our proposed study will compare the microbiome of commonly used bedding strategies by identifying taxa that are associated with healthy udders and by characterizing strategies associated with reduce incidence and prevalence of mastitis.

Table 1. Bedded-pack shares health-associated fungi with teat skin and milk. Fungal genera ≥5% of total relative abundance in teat or mammary gland also present in bedded pack. Green cells are shared taxa lower in infected glands, thereby putatively associated with the healthy udder; Yellow cells are bedding taxa increased in infected glands.

Genera	Healthy	Infected	Fold Change	**Pack**
Teat	Mean percent teat skin community			
Cryptococcus	7.6%	2.7%	↓ 2.8	< 0.1%
Penicillium	6.8%	6.4%	↓ 1.1	0.4%
Wallemia	4.4%	5.6%	↑ 1.3	< 0.1%
Debaryomyces	17.8%	19.2%	↑ 1.1	3.7%
Mammary	Mean percent mammary community			
Debaryomyces	1.4%	11.6%	↑ 8.3	3.7%
Aspergillus	0.2%	22.0%	↑ 110	1.0%
Aureobasidium	0.0%	5.8%	↑	< 0.1%
Penicillium	23.3%	13.5%	↓ 1.7	0.4%

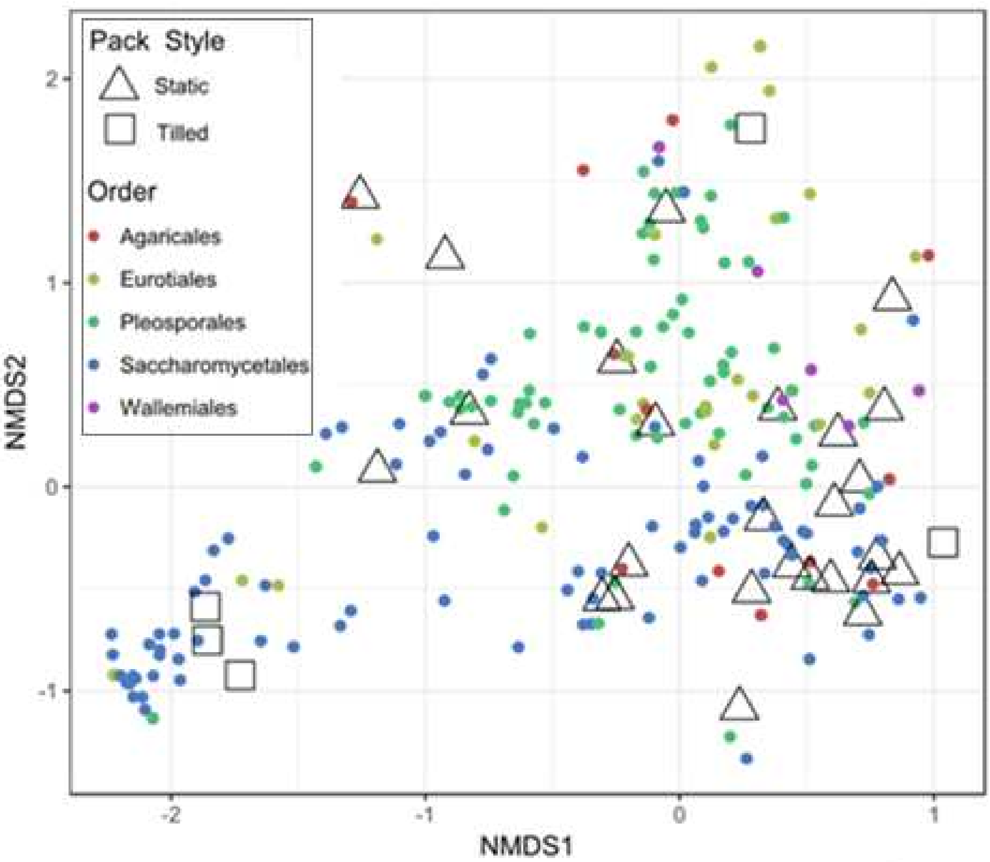
Neher et al. unpublished.

Rationale and Significance

The project will gather survey data on the current practices of bedding on organic farms, and empirically evaluate relationships between bedding microbiomes and their impact on animal health and milk yield. A member of our advisory panel suggests that **prevention of mastitis is the first line of defense in producing high quality milk**.

Members of our advisory panel indicate that the project will provide a valuable budget tool, and that our research extends beyond animal welfare and economics, to address an environmentally friendly method of managing manure as a solid rather than as a liquid. Our approach is interdisciplinary, applying methods and concepts from animal/dairy science, microbiology, epidemiology, and agricultural economics. One advisor suggests **organic dairy farmers are "intensely interested in the impact of different bedding strategies on udder health."** Another advisor says she regularly fields questions from farmers regarding the relative costs associated with bedded pack compared to the benefits of animal health and milk yield. The project will provide practical results to farmers to make decisions on renovation of existing housing, construction of new housing and management of existing or new housing.

Figure 3. Nonmetric multi-dimensional scaling ordination of Bray-Curtis dissimilarity matrix of top fungal orders. Closer spacing of taxa represents similar abundance between samples. Closer spacing of samples represents similar abundances of taxa within those samples.



Culture independent molecular tools present an opportunity to understand the potential of the intramammary microbiome to resist dysbiosis and limit risk of infection. Organic dairy farms, with limited mastitis treatments available, stand to benefit from innovations for mastitis control. Bedding strategy creates a unique microbial environment, which may differentially modulate the udder microbiome, offering farmers a management tool to foster an udder microbiome that resists pathogen colonization and infection. Our aim is to assess how current bedding practices and mastitis control techniques affect the prevalence of mastitis and milk quality on northeastern organic dairies (OREI 2018 **priority 1**). We will investigate differences in the microbial community that impact infection and resistance to mastitis, and identify improved management techniques that increase animal welfare, milk yield and quality (OREI 2018 **priority 6**). We aim to engage extension personnel during each objective of the project to build relevant management tools that will be disseminated by researchers and extension personnel via multiple industry-wide web, paper and in-person media outlets. We will develop management guidelines that describe how currently used bedding practices can be adjusted to foster beneficial microbes and inhibit pathogens (OREI 2018 **priority 2**). Furthermore, this study is potentially transformative in establishment of new tools for risk assessment and disease prevention that are based on the integration of culture-dependent and culture-independent approaches to mastitis epidemiology.

Novelty

The advent of DNA-based methodology allowed characterization of whole microbial communities. Most prior microbiome work has focused on bacteria using traditional cultures or amplicon sequencing of one of the variable regions of the 16S gene (Lima et al. 2017). These techniques are employed for BTM (Oikonomou et al. 2014), teat skin (Falentin et al 2016) and individual cow milk samples (Kuehn et al. 2013). These methods overlook fungi known to produce antibiotics naturally. Our preliminary research finds greater abundance of *Penicillium*, *Rhodotorula* and *Sistotrema* fungi in healthy compared to mastitic milk. To our knowledge, our research will be the first to characterize fungal community composition in housing and milk products with amplicon sequencing (Vacheyrou et al. 2011). The presence of bacterial DNA in milk samples could be due to dead bacteria or bacterial fragments (Rainard 2017). We will take the next step of determining whether bacteria and fungi are alive by removing relic or dead DNA using a propidium monoazide method (Carini et al. 2016, Erkus et al. 2016). Furthermore, we will be among the first to focus on gene function by employing shotgun sequencing techniques (Bhatt et al. 2012, Keeney et al. 2014) which adds knowledge and understanding of mechanisms by testing the hypothesis of dysbiosis.

Given our goal to identify management practices integrating microbiological risks and benefits of winter housing strategies, it is important to use culture-independent molecular tools to characterize the potential for a resilient microbiome, resisting change and preventing infection. The indigenous microbiome may modulate the potential for opportunistic bacteria or fungi to become pathogenic, preventing infection. This study seeks evidence of a resilient indigenous microbiome, and, by testing the hypothesis of dysbiosis, we test the possibility that farmers can use bedding strategy to prevent mastitis and develop healthy microbiomes in their animals and barns.

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Approach

Objective 1 – Assess current bedding management practices, mastitis management, animal health and milk quality on northeastern organic dairy farms.

Experimental design: We will enroll 40 organic milk producers to collect data from in-person interviews, on-farm observations, and samples for analysis. Given the focus of the study on bedded-pack loose-housing systems, which are a relative small proportion of all organic herds in Vermont, we will recruit the study population as a non-proportional stratified random sample of surveyed farms. The stakeholder advisory panel will assist in determining criteria for stratifying herds prior to selection. Our preliminary data on bedded pack management and a review of published research and extension literature will serve as a guide to identify key stratification and selection criteria, with the goal of minimizing the number of enrollment and stratification criteria. We aim to enroll 10 organic dairy herds for each of four common bedding/housing strategies: mechanically aerated bedded pack, static bedded pack, individual tie-stall, and individual free-stall housing systems. The state certifier agency, Vermont Organic Farmers LLC, will assist by providing a comprehensive list of producers, however, there is no database that includes housing strategy for each farm. To identify our target population of 40 farms, we will communicate with other research teams in Vermont that are currently engaged in research on Vermont organic dairy herds (S. Greenwood and H. Darby) and conduct a brief industry wide survey administered using a combination of telephone, mail and internet. Short in-person interviews will occur at regional farmer meetings (See Outreach Plan, part 1, Conferences, p. 19). Correspondence with farmers and industry representatives suggests that our statewide survey is feasible and an opportunity to strengthen their industry by exploring techniques for reducing mastitis.

Data Collection: To capture information on bedding management practices, facility design, herd demographics, and mastitis management practices on the 40 target farms, a comprehensive questionnaire about bedding management practices and mastitis management will be developed based on prior survey tools developed by other research teams in the US (http://milkquality.wisc.edu/organic-dairies/project-c-o-w/) and Canada (S. Dufour, personal communication). In collaboration with the stakeholder advisory panel, we will develop survey questions related to housing, bedding management, mastitis incidence, mastitis treatments, BTM SCC, BTM bacteriology, animal hygiene and lameness. PI Barlow's lab will visit each enrolled herd once to conduct the interview, complete the management practices questionnaire, make on farm observations, and collect samples for laboratory analysis.

Data Analysis: Survey results will be compiled to assess bedding strategies and mastitis prevalence on organic dairy farms. Bedding strategies and outcomes (prevalence of mastitis and BTM quality) will be compared among three general housing and bedding strategies (tie-stall, free-stall, and bedded pack). If survey data suggests that distinctions between groups be adjusted, or groups added (e.g., mechanically aerated vs. static bedded pack) the additional variables will be added to alternative models. Potential associations between each variable and the outcome measures will be quantified through a model building approach. Variables found to be associated with the outcomes in a univariate model at $p \leq 0.20$ will be included for subsequent multivariate analysis. We will test for collinearity among predictor variables and outcomes to develop a structural equation model that quantifies direct and indirect pathways. Predictor variables will include udder hygiene, BTM quality and bedding strategies. Outcome variables will be incidence and prevalence of clinical and subclinical mastitis (Barlow et al. 2013). A multivariate modeling approach will be used to test associations between milk quality and mastitis outcomes and bedding strategies (forced into all models) while accounting for the effect of covariates identified as significant predictors (Schreiner & Ruegg 2003). A significance threshold $p \leq 0.05$ will be used to assess significant associations within final models.

Stakeholder Advisory Panel Meeting: The initial complete stakeholder advisory panel consultation will occur at an in-person 3-hour meeting during the survey development to guide questions toward industry relevant data and provide background on potentially confounding management practices or industry wide events. The meeting will be scheduled at the March 2019 Vermont Organic Dairy Producers Conference to make attendance easier and to inspire participation in the project among producers.

Outcomes: We will report results characterizing bedding strategies and differences in factors contributing to mastitis prevalence and milk quality to help producers contextualize and adapt their own practices within the northeast organic dairy industry. Results from research Obj. 1 will be used to identify and recruit farms for participation in Obj. 2 of the study.

Objective 2 – Improve our understanding of mastitis epidemiology, bedding microbiology and characteristics, and bedding management practices.

Experimental Design: The goal is to characterize how winter housing/bedding strategies identified in Obj. 1 affect the prevalence of mastitis, milk quality, the characteristics of bedding, and structure and transmission of the bedding and udder microbiome. While it would be ideal to test all bedding strategies, time and budget constraints limit the feasibility of this approach. Instead, we plan to test the most common overall bedding strategy and the most common bedded pack strategy as suggested by the industry wide survey conducted in Obj. 1. We will recruit five farms from each of two selected bedding strategies. Criteria for study farms are that they exhibit common characteristics of their group as suggested by mid-range prevalence of mastitis and BTM quality and shared management practices. The stakeholder advisory panel will consult on differentiation of bedding strategy groups, importance of potential treatment groups to the industry and key features of typical farms to be assigned to each group.

Recruitment will occur via phone or email. Participants will allow researchers access to bedded pack and cows at milking time once per month for six months. Additionally, farmers will be provided with record keeping sheets to collect a second winter of data on housing/bedding strategy and mastitis as described in Obj. 1. A small stipend will compensate farmers for time spent assisting researchers and record keeping. We do not view recruitment of five farms within each bedding strategy as challenge in light of the noted level of engagement in the farming community. Most data collection methods are minimally intrusive and do not require additional producer involvement on sampling day. Sampling will occur once monthly between November 1 st and May 1. This is the period during which cows are most unlikely to access pasture and spend most of their time in the winter housing/bedding environment.

A population of thirty-five (35) cows per herd will have individual milk samples collected from each quarter. Cows will be stratified and selected randomly within each stratum within each farm to prevent bias from individual cow factors such as parity, days in milk, and SCC at enrollment. Individual cow or quarter SCC will be determined by processing samples through a local commercial testing laboratory or by use of DeLaval cell counter. Only herds with 35 or more cows will be enrolled. Sample size is determined for the outcome mastitis incidence (new clinical and subclinical mastitis cases) where the proportion of new cases in bedded-pack herds is conservatively estimated as 10% and the proportion in tie-stall herds is 20% (Fig. 2). For a comparison of proportions of new mastitis cases, adjusting for within herd clustering (Barkema et al. 1997) and multi-variable models with as many as 6 covariates and a type 1 error rate of

α=0.05 %, and power, 1 –β=0.80%, a sample size of 684 quarters or 171 cows for each bedding type is estimated to be sufficient. To be cost effective, we will sample microbiomes for combinations of bedding strategy and health status at the quarter level of individual cows. SCC and culture assay will be used to define four classes of udder health: (1) "always healthy" (SCC is < 200,000 cells/ml for each sample time + no pathogen), (2) "became infected" (SCC is < 200,000 cells/ml before increasing to ≥ 200,000 cells/ml + pathogen), (3) "became infected and recovered" (SCC returns to < 200,000 cells/ml + no pathogen after increasing to ≥ 200,000 cells/ml + pathogen), and (4) "always infected" (SCC ≥ 200,000 cells/ml + pathogen identified for each sample time). A stratified random subsample of *N*=300 quarters from each bedding strategy will be submitted marker gene sequencing (10 samples per health category x 4 categories x 6 months, plus 2 sampling and processing negative control samples per month).

Herd Level Data Collection: Herd level data will be collected monthly on BTM and bedding at the time of cow sampling. In bedded-pack and free-stall barns, five 3.75-L samples will be collected along random transects at each sampling date. In bedding deeper than 20 cm, samples will be a composite of the upper 20-cm. In tie stall barns, a composite 1-L sample will be assembled from five locations along a random transect in individual stalls of sampled cows. Samples will be aggregated on-site in a sterile container and a composite 3.75-L sample formed. Temperature and percent oxygen will be measured at time and location of sampling. Samples will be transported to the lab in coolers for same-day processing. A composite sample will be formed for each farm each month. Composite samples will be resampled at approximately 1-cc and ground using a mortar and pestle. PI Neher's Lab has routinely performed bedding sampling on multiple farms and bedding strategies throughout Vermont (Fig. 3).

DNA extraction and sequencing of marker genes: Prior to DNA extraction, microbiome samples will be exposed to propidium monoazide (PMA) to remove extracellular and lysed cell DNA (Carini et al. 2016). Briefly, PMA is added to an aliquot of ground bedding suspended in phosphate buffered saline, vortexed in the dark before repeated 30s/30s light/dark exposures with a 650 W halogen bulb. Removal of "relic DNA" is a recently established technique that has been used in diverse microbial environments, including soil and cheese (Nocker et al. 2007, Erkus et al. 2016, Carini et al. 2016). The method reduces the potential of dead bacteria or extracellular DNA to skew community composition data. The technology works by preventing amplification of relic DNA during the PCR step of marker gene sequencing. The Neher Lab has performed internal proof of concept experiments confirming its efficacy at preventing lysed-cell DNA from amplification (Readyhough, Weicht & Neher, unpublished data). PMA based removal of relic DNA is a proven technology that can help elucidate critical current questions in the field concerning the accuracy of finding living bacteria or fungi within the mammary gland.

After extracellular and lysed cell DNA is removed, samples will be frozen at -80C until later DNA extraction using Qiagen DNeasy PowerSoil kit following the manufacturer's instructions with slight modifications: an incubation for 10 min at 65 C and 2 minutes vortex in the bead tubes (Lauber et al. 2009). DNA extraction is regularly performed by PIs Barlow and Neher's labs. The Quiagen DNEasy PowerSoil Kit has been used successfully on bedding, teat and milk samples by our labs (Neher et al., Barlow et al. unpublished).

Extracted DNA samples will be frozen at -80C and shipped overnight express on dry ice to the University of Colorado Next Generation Sequencing Facility. Noah Fierer's (member of advisory panel) group will prepare samples by pooling equal aliquots for amplicon PCR and sequencing on the Illumina MiSeq platform (Emerson et al. 2015). Marker genes are amplified using 515F and 806R primers for V4-V5 region of bacterial 16S rRNA genes for bacteria and archaea and ITS1F and ITS2 primers for fungal ITS1 region. Cleaned and quality filtered reads are dereplicated and clustered at 97% nucleotide identity into representational OTU sequences using the UPARSE pipeline (Edgar 2013). The PIs have experience with these methods in prior collaborations with Fierer Lab (Neher et al. 2013, Cutler et al. 2018, Fierer letter of support).

BTM samples (approximately 200 ml) will collected from the top of the bulk tank using sterile single-use polystyrene sample collection containers with detachable handles (SterlinTM DippaTM #192, Thermo Scientific), following 5 minutes of bulk tank agitation. All milk samples will be collected by laboratory personnel, transported on ice to the laboratory, held in the lab under refrigeration, and cultured within 24 hours of sampling. SCC will be measured using a DeLaval Cell Counter DCC (DeLaval-USA, Kansas City, Missouri).

Cow Level Data Collection: Teat skin and intramammary milk will be collected from all quarters on selected cows monthly at the same time point as herd level sampling. Teat end swabs of each quarter of the udder will be collected using sterile flocked swabs (Copan Diagnostics) wetted with transport medium and wiped across the teat end apex. Teat swabs will be collected immediately prior to preparing the teats for intramammary milk sampling. Teat swabs will be placed in 3 ml of transport medium and placed on ice. Teat swabs in transport vials will be vigorously shaken for 5 minutes at 2500 rpm and aliquots of the suspension will be removed for aerobic culture. Serial 10-fold dilutions of the teat swab suspension will be plated on non-selective and selective media to quantify total aerobic, *Streptococcus* spp., *Staphylococcus* spp. and coliform bacteria counts. An aliquot of the remaining teat swab suspensions will be submitted to the extracellular DNA removal protocol before freezing both the processed aliquot and unprocessed remaining sample at -80C for community DNA sequencing. Removal of extracellular DNA, extraction, PCR, sequencing and initial bioinformatic pipeline will occur as described for bedding and BTM samples.

Quarter milk samples of all enrolled cows will be collected monthly by the research team using standard aseptic methods. Farmers will collect individual quarter milk samples from all cases of clinical mastitis occurring during the study and store them frozen until collected monthly by researchers. Participating farmers will record all mastitis events and treatments on data forms provided by the research team. Milk samples will be cultured using standard established methods for mastitis bacteriology to identify aerobic pathogens causing mastitis(Middleton et al. 2017). Isolates will be passed in cultured for purity and stored frozen at -80C in tryptic soy broth with 15% glycerol or on Microbank™ beads (Pro-Lab Diagnostics) until further analysis. Presumptive bacterial species identification will be by biochemical standard methods from isolates organisms grown in pure culture, and confirmed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) in collaboration with Dr. P Adkins (member of advisory panel). MALDI-TOF is emerging as an inexpensive reliable method to type bacterial species isolated from milk, dairy cattle and farms (Savage et al. 2017, Cameron et al. 2018). In addition, milk samples for DNA extraction will be collected via double catheter to prevent contamination of sample with microbes located in the teat canal or teat surface. Quarter milk SCC will be measured as described for BTM. Secondary aliquots of quarter samples will be used in inhibition assays in Obj. 3, described below. An aliquot of the remaining milk samples will be processed similarly to teat samples for culture-independent analysis. Mammary milk and teat skin sampling technique has been routinely performed by PI Barlow's Lab. The double catheter technique for collecting milk prevents the contamination with organisms that might reside in the teat canal or on the exterior of the teat. Typical aseptic sampling technique risks contamination. This is the most invasive technique used in the study but does not appear to cause cow discomfort and can be conducted directly before milking with little participation from the farmer. Teat skin microbiome sampling is quick, minimally invasive, and inexpensive. Cow-side sampling is the most time intensive on-farm sampling activity. PI Barlow, graduate student and trained undergraduates will complete cow-side sampling to ensure that sampling is sterile, efficient, and does not impact farm activities.

Statistical Analysis: OTU tables will be normalized and singletons removed. Differences between microbial communities will be quantified by converting square-root transformed data to construct Bray-Curtis dissimilarity matrices. Patterns of community composition will be visualized using Non-metric multi-dimensional scaling (NMDS) ordination. Subsequently, effects of bedding strategy, udder health status, SCC and sample type will be quantified by permutational analysis of variance. The effect of bedding strategy on abundance of taxa in bedding, milk and teat skin will be tested using Kruskal-Wallis tests. False discovery rate adjusted P values will be reported to correct for multiple comparisons. Proportion of taxa shared between sample types and BTM will be calculated and compared between bedding strategy using multivariate analysis of variance techniques. All statistical computing will be performed using R (R Core Team 2017).

Outcomes: We will publish results comparing and prevalence of clinical and subclinical mastitis between bedding strategies and associations between bedding strategies and bedding characteristics, specific taxa, and high or low herd level rates of mastitis. We will publish recommendations and guidelines describing practices related to lower BTM SCC and bacteria counts and improved udder and flank hygiene scores and prevalence of lameness. Completion of Obj. 2 will provide SCC, microbiome and culture data that will be used to select samples for further analysis of microbial function in Obj. 3.

Objective 3 – Exploring microbial community function of the intramammary gland and how it impacts susceptibility to mastitis.

The goal is to refine our understanding of microbial behavior in the mammary gland and how it impacts susceptibility to mastitis. We will conduct two complementary experiments designed to (1) seek evidence for inhibition of pathogens by the mammary microbiota and (2) suggest possible taxa and mechanisms by which inhibition could occur.

Experiment 1: The ability of bacterial or fungal organisms isolated from milk or skin swabs to inhibit mastitis pathogens will be evaluated in simultaneous antagonism assays (Christensen et al. 2016, De Vliegher et al. 2004). Briefly, to detect inhibitory activities between multiple isolates of the two species agar plates are inoculated on one side with a streak of a potential inhibitor organism, incubated for 18 to 24 hours, then the agar plate is flipped and the reverse side inoculated with a lawn of an indicator organism, and again incubated overnight. Evidence of growth inhibition of the indicator organism is observed by reduced growth in zones adjacent to the inhibitor organism. In a modification of this procedure, whole milk, skin swab, and bedding sample diluted suspensions will be plated as potential inhibitors and individual colonies growing from these suspensions that inhibit the indicator organisms will be collected and identified. Indicator organisms will include a panel of mastitis pathogens (*S. aureus, Streptococcus uberis, Streptococcus dysgalactiae, Staphylococcus chromogenes, Escherichia coli, and Klebsiella pneumonie*). Frequency of inhibitor organisms will be compared among herds by bedding type. Individual inhibitor organisms will be identified to the species level and the diversity of inhibitors identified will be compared among herds by bedding type. The relationship of phylogeny and metabolic profile to inhibition gradient will be analyzed using canonical correspondence analysis.

Experiment 2: To compare microbial functional gene groups between healthy and mastitic cows, SCC and plated culture assays from Obj. 2 will be used to group quarters into health levels “always healthy” and “always infected” as defined above. A quarter with elevated SCC and no evidence of cultivable pathogen will be defined as “always infected” if the community sequencing result suggests dysbiosis evidenced by dominance of a single organism in the mammary gland. To compare microbial genetics between each group, aliquots from 20 randomly selected quarters from each group (2 treatments x 20 cows = 40 samples) will be diluted in saline solution and filtered with a 3 micron nitrocellulose filter to remove somatic cells (Bhatt et al. 2012). DNA will be extracted via protocol described above and refrozen at -80C for shipment to University of Colorado Next Generation Sequencing Facility for shotgun metagenomic sequencing using protocols described by Brewer & Fierer (2017).

Shotgun metagenomic sequencing of fungi and prokaryotes: We are most interested in gene-groups such as virulence, disease and defense and stress response (Bhatt et al. 2012). Given the proof-of-concept nature of this objectives, and expense of shotgun sequencing, we plan to run 40 samples (2 treatments x 20 cows). Shotgun sequencing will be run on the Illumina Hi-seq at University of Colorado Next Generation Sequencing Center. Given the complex interpretation (Callahan et al. 2016, Singer et al., 2016), we will rely on the Fierer lab to provide initial data quality screening and filtering and assist with choice of pipelines and interpretation of the data. Normalization, phylogenetic reconstruction, and gene annotation of unassembled, merged, quality filtered sequences will be performed via the MG-RAST online processor, which accesses SEED-based subsystems data to group genes by function (Meyer et al. 2008). We will have considerable metadata to raise our priority level for using MG-RAST (F. Meyer, personal com.). Relationships between taxa or gene groups and area of inhibition (Experiment 1 of Obj. 3) will be tested using regression analyses.

Outcomes: The experiments will avail organic farmers of the most cutting-edge tools with which to inform management decisions concerning animal health and milk quality. We will publish comparisons of differential functioning of the microbiome in healthy and mastitic animals. We will characterize the structure and function of the microbiome that resists infection and will develop recommendations and guidelines describing bedding strategies most likely to help promote this functionality.

Construction of management practice guidelines: Completion of all research objectives will provide empirical data to establish a set of management practice guidelines that recommend strategies to reduce mastitis risk on organic dairy farms. To enable farmers to make more informed management choices that help prevent mastitis, guidelines will include easy to interpret diagrams of pathogenic and beneficial microbial flow between bedding, animal and bulk tank, with differences noted by bedding type and prevalence of mastitis. The *stakeholder advisory panel* will provide input on relevancy and feasibility of recommendations during a final in-person meeting. Because usefulness to the producer is an important outcome of our work, outreach specialists co-PIs Alvez and Colby will write articles and bulletins, and consultant Flack will assist in writing guidelines that translate research into management practices. PIs Neher and Barlow have a history of frequent work with farmers and industry support personnel. The guidelines will be structured by bedding strategy, outlining: (1) popularity of current bedding practices in use by the industry and key aspects of each (Obj. 1). (2) mastitis prevalence for identified bedding strategy groups (Obj. 1). (3) Key differences (such as pathogens or beneficials) in bacterial and fungal communities between most common bedded pack and most common overall bedding strategy (Obj. 2). (4) Key differences in bacterial and fungal community between healthy and mastitic cow quarters (Obj. 2). (5) Effect of bedding strategy on SCC, mastitis rates, animal hygiene and milk quality (Obj. 2). (6) Evidence for inhibitory effect of the milk microbiome and the effect of bedding strategy on fostering a resistant microbiome (Obj. 3). In addition, we will work with the dairy management economic advisor on the team to construct a bedding-planning budget model that incorporates mastitis cost parameters based on observed data from Obj. 1 & 2. The economic decision tool will be structured for farmers or their advisors to use when comparing bedding management scenarios, evaluating alternative bedding material purchases, and generating budgets for facilities replacement or renovation.

Outreach Plan

Continued engagement of the stakeholder advisory panel at each stage of the project will ensure relevance for farmers and generate interest within the industry. **Outreach Action 1** will focus on generating a portrait of emerging and established bedding strategies on northeast organic dairy farms and the association with prevalence of mastitis, animal hygiene and milk quality to help producers contextualize and make informed decisions concerning their own practices. **Outreach Action 2** will focus on communicating with farmers on how pathogenic or health associated microbes are transmitted between bedding, teat and cows and how this differs between bedding strategies. **Outreach Action 3** will focus on development and dissemination of integrated management practices guidelines that have as the overall goal using bedding management to foster a cow microbiome that reduces exposure to pathogens. In conjunction with our collaborators at UVM Extension and the Center for Sustainable Agriculture, in **Outreach Action 4** we will disseminate information via the following activities:

1. <u>Conferences</u>: (a) Vermont Organic Dairy Conference (mid-March annually): We plan to participate in this conference in year 2 (describe research Obj. 1 & 2) and year 3 (describe research Obj. 3 and integrated results). Many attendees of this conference are stakeholders and timely updates are critical. (b) Northeast Organic Farming Association of Vermont Conference (mid-February annually): We plan to share integrated results at this conference in year 3. (c) Vermont Grazing and Livestock Conference (mid-January annually): We will participate in this conference in years 2 and 3 to present ongoing results.
2. <u>Newsletters</u>: At the conclusion of the study integrated results and practice management guidelines will be submitted to newsletters: (a) *On Pasture*: online, dedicated to translating scientific research into farm management practices, reaching 100,000 readers per month, (b) Northeast Organic Dairy Producers Alliance Newsletter, (c) ATTRA (A National

Sustainable Agriculture Assistance Program) Newsletter, (d) Northeast Pasture Consortium Newsletter

3. <u>On Farm Events</u>: We anticipate hosting two on farm events at participating farms to highlight results from research Obj. 1 & 2 (year 2) and integrated research results and management recommendations in year 3. Meetings will occur in winter months while bedded-pack is in place.
4. <u>Extension Bulletins</u>: Publication of management practice guidelines (similar to Gilker et al. 2012).
5. <u>Student Involvement</u>: The study will provide funding for one graduate, and at least three undergraduate students. The graduate student will be a PhD student who will be engaged in all aspects of data collection and analysis. The undergraduate students will assist in sample and data collection and laboratory analysis, working during the academic year and summer creating an exciting undergraduate experiential learning opportunity. They will disseminate information regarding the project through oral and poster presentations at on-campus undergraduate research workshops and conferences.
6. Publications in scientific peer-reviewed journals: We anticipate producing three peer-reviewed articles from the research completed during this study. Publications will focus on characterizing currently used bedding practices on organic farms (Obj. 1), comparing epidemiology of mastitis between common bedding strategies (Obj. 2), and comparing structure and function of microbiome between mastitic and healthy quarters (Obj. 3).

Expected Results and Usefulness of Research

A critical goal of our work is to provide useful information to producers about how bedding strategies may affect how beneficial and pathogenic microbes are transmitted between animals, bedding and bulk tank with the ultimate goal of reducing mastitis risk and producing higher quality milk. To this end, we plan to create an easy to read set of management guidelines that will be disseminated via multiple media types and outlets that serve the organic dairy industry. Management guidelines will (1) characterize current bedding practices and farmer reported prevalence of mastitis, (2) report differences in quantified mastitis risk between common bedding strategies, (3) characterize microbial transmission in common bedding strategies, (4) examine practices that develop a microbiome that reduces pathogen exposure. These guidelines, along with detailed presentations for each objective of the study will be shared with organic dairy farmers, organic technical advisors, and organic dairy cooperatives and published in multiple media platforms. We anticipate our research results will be used by all stakeholders to reduce mastitis risk and produce higher quality milk, creating more productive and financially sustainable organic dairy farm systems in the northeast.

Pitfalls and Limitations

Our team brings a proven expertise, skillset and familiarity with the organic dairy industry and epidemiological field studies of dairy cattle mastitis that minimizes the chance of pitfalls in the organization, data collection and analysis of the proposed study. We are also engaging with scientific and industry advisors to identify and minimize pitfalls during the project. Under research Obj. 1, the use of a non-proportional stratified random sample does not capture the true overall estimates for the outcomes compared to the alternative of a simple random sample of willing participants from all organic herds in Vermont. Given that our preliminary data indicates herds using pack-housing systems are relatively infrequent compared to tie-stall and free-stall herds, a simple random sample runs the risk of having limited observations from bedded-pack herds. The stratified random sample we propose divides the population into strata based on housing and bedding strategies before randomly identifying potential participants. This has the advantage of ensuring that bedded pack herds are well represented in the study population but limits generalized industry-wide inferences. Likewise, to obtain the depth of sampling necessary to build statistically accurate representations of the microbial community, it was necessary to restrict treatment groups to two common bedding strategies. This limits the scope of management variation evaluated but provides a very strong platform on which to compare the healthy and mastitic microbiome. By choosing the most common bedding strategies, we hope to make bedding strategy specific recommendations that will benefit the greatest number of producers directly, while applying the most cutting-edge molecular tools to analyze mastitis data that will benefit all organic producers.

Weather and changing climate patterns can potentially create atypical conditions that could skew results. The northeast has been experiencing prolonged warm weather in the fall and increased frequency of massive precipitation events that affect pasture usage and need for housing/bedding. Variation during the 6-month winter sampling period may be unavoidable, so we will incorporate constant environmental monitoring within the dairy barns using data loggers that record ambient temperature and humidity at frequent intervals (as frequent as every minute) to account for environmental variation. By conducting a preliminary herd visit for the herds under obj. 1 we will obtain data to make a comparison between subsequent years.

A challenge with shotgun sequencing is there are multiple pipelines and no set guidelines for specific genes to follow. The sequencing is relatively simple, but the interpretation is challenging. Therefore, we invited Noah Fierer to serve as a consultant to assist in that capacity (Brewer & Fierer 2018).

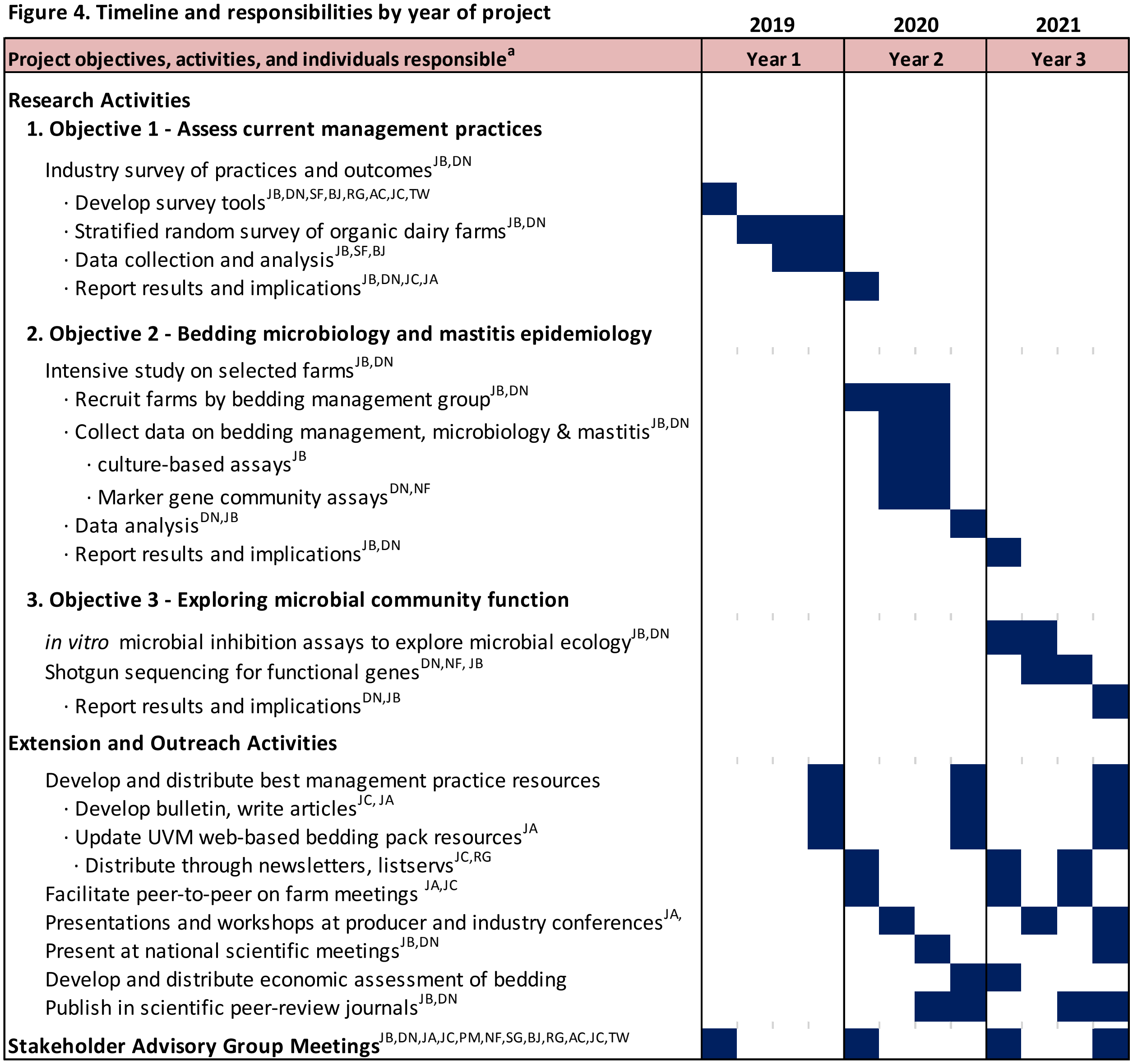
Hazards

Hazards are associated with physical injury during on-farm studies and exposure to potential opportunistic human pathogens during laboratory analysis. To minimize hazards associated with animal research and handling, any research personnel involved with on-farm activities will be required to complete online IACUC trainings and quizzes. Personnel will also receive generally environmental safety training for on-farm activities through UVM Environmental Safety Management, and will be trained on farm specific hazards or considerations by the producer prior to conducting research on their farm. To minimize laboratory hazards, all personnel involved with laboratory analysis will be required to complete all training in accordance with UVM biosafety regulations. Material Safety Data Sheets and lab safety protocols are available to all personnel to become aware of hazards specific to each lab. The Barlow lab, where microbiological studies will be conducted, is a BSL-2 laboratory with all appropriate microbiological standard operating procedures and Institutional Biosafety Committee approvals.

Timeline, Roles and Responsibilities

Data management is the responsibility of PIs Barlow and Neher. The PIs will supervise weekly project meetings during their active times on the project to ensure data management requirements are met. The responsibilities of other participants (key personnel, collaborating scientific and outreach advisors and other advisory panel members) are identified in the project timeline (Fig. 4).

Figure 4. Timeline and responsibilities by year of project



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Key personnel and Project Consultants: Juan Alvez (JA), Jennifer Colby (JC), Pamela Adkins (PM), Noah Fierer (NF), Sarah Flack (SF), Sarah Godden (SG), Brian Jerose (BJ), Rachel Gilker (RG)
Remainder of advisory panel: Annie Claghorn (AC), John Cleary (JC), Tyler Webb (TW)