Northern NY Agricultural Development Program
2016 Project Report

Speciation and Quantification of Streptococcus-like Bacteria from Bulk Tank Milk, Bedding, and Teat Swabs Pre- and Post-milking from Dairy Herds in Northern New York

Project Leader:

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Cooperating Producers:

St. Lawrence County	Jefferson County	Lewis County
Adon Farm	Butterville Farm	Moserdale Dairy
Brandy View Farm	CTS Dairy	**Franklin County**
Chambers Farm	Deer Run Dairy	Vincent Farm
Kelly Farm	Doubledale	**Clinton County**
McKnight River Breeze Farm	Hy-Light Farm	Adirondack Farm
L. T. Smith and Sons	North Harbor Farms	
Stauffer Farm	Sheland Farm	
Woodcrest Dairy	Windsong Dairy	
Twin Mill Farms		

Background:

Environmental organisms cause the majority of clinical mastitis in herds with low bulk milk somatic cell counts. Environmental *Streptococci* (non-*agalactiae*) can contribute significantly to clinical mastitis, although the organism responsible and prevalence can

vary significantly between farms (Gröhn, et al., 2004; Hogan, et al., 1989; Scillieri Smith NNYADP grant, 2014).

Management of intramammary infections (**IMI**) caused by environmental organisms focuses on prevention by reducing the potential for bacteria to come in contact with the teat end and enter the teat canal (Smith et al., 1985). Exposure can be minimized by improving cow hygiene through stall and bedding management and focusing on teat end cleanliness before milking (Elmoslemany, et al., 2008; Bartlett, et. al., 1992).

Streptococcus uberis has been identified on bovine teats, udder surface and other superficial locations (Cullen, 1966; Bramley, 1984) and may act as a potential reservoir for new infections. Bedding and feces have also been shown to serve as an environmental reservoir for this organism (Bramley, 1982). Research has shown an association between bacterial contamination of teat skin with *Streptococcus* species and *Streptococcus uberis* and the levels in bedding over time (Zdanowicz, et al., 2004; Paduch, et al., 2013).

Recent research in Northern New York focusing on environmental causes of intramammary infections, specifically by *Streptococcus*-like bacteria, has shown that the group of pathogens previously identified as "other *Streptococci*" includes *Lactococcus* species. This research also identified *Lactococcus* as a major mastitis pathogen on some farms (Werner et al., 2014; Scillieri Smith, NNYADP grant 2014 and 2015).

Data from the Northern New York Agricultural Development Program-funded project (n=143 farms) showed that although *Lactococcus lactis* was in 24% of the mastitis milk samples in the *Streptococcus*-like category of bacteria, it was only found on 19 farms (13%). This is a noteworthy contrast to *Streptococcus uberis* which was identified in 32% of samples and on 76 farms (53%) or *Streptococcus dysgalactiae* which was identified in 33% of samples and on 35 farms (24%). In fact, for the 6 farms with the highest number of samples included in the project, each farm appeared to have a predominant organism (either *Streptococcus dysgalactiae* or *Lactococcus lactis*) causing more than 50% of the mastitis caused by Gram positive, catalase negative cocci (**GPCN**) organisms.

Because *Lactococcus* mastitis appears to cluster on some farms, the question of either farm-specific risk factors or contagious spread of *Lactococcus* species has required further investigation.

Because *Lactococcus* spp. have previously been grouped with other *Streptococcus*-like, gram positive, catalase negative organisms in bulk tank milk, bedding and other environmental cultures, knowledge of their presence in these samples is limited. Currently, *Lactococcus* spp. are presumed to act as environmental pathogens and would be managed accordingly. However, because *Lactococcus* has not been previously considered a major mastitis pathogen, its occurance in bedding and other environmental sources which can put cows at risk for IMIs has not been investigated.

Additionally, *Lactococcus lactis* has been identified as a major mastitis pathogen, putting cows at risk for both chronically high somatic cell counts and culling on some NNY farms. This research positions *Lactococcus lactis'* role in IMIs on dairy farms for further investigation.

Preliminary work with the NNY farm samples showed that *Lactococcus* spp. can be cultured from both bedding and bulk tank milk when present as a major pathogen on the farm. Evaluation of *Lactococcus* species in bedding samples, on teat skin, and in bulk milk on farms with known levels of *Lactococcus* spp. mastitis may help to explain the interaction between environmental exposure to *Lactococcus* spp. (e.g. bedding, teat skin) and IMI (e.g. bulk milk). Additionally, this information may help identify whether bulk milk can be used as a screening tool to identify herds with *Lactococcus* spp. IMIs. This is important because *Lactococcus* spp. appears to be an emerging mastitis pathogen.

Methods:

In 2016, ten farms with >1% *Lactococcus* species from clinical mastitis (**CM**) cultures (**LP Herds**) and ten with ≤1% *Lactococcus* species in cultures from CM cows (**LN Herds**) were selected. Farms were included only if they regularly submitted milk samples from cows with CM in order to be able to assess what mastitis pathogens were present in the herd. Herds in both groups were balanced for size.

Between June and December of 2016, environmental samples were collected from all farms, including a bulk tank sample, bedding samples (1 new and 3 to 4 used) and teat swabs from 15 fresh cows.

Bedding samples were taken from the fresh pen if possible; new bedding samples were taken at the time of application, and used bedding samples were taken from the back 1/3rd of the stall, covering a 2 foot by 2 foot area, harvesting the top layer of bedding material.

Teats were swabbed using BBL CultureSwab Plus (Becton, Dickinson and Company, Franklin Lakes, NJ) at 3 time points during milking preparation: upon entry into the parlor, after pre-dip was applied and removed (after prep), and after milking, before post-dip application). Teats ends were swabbed using a cross pattern with two 1cm long swipes crossing at the teat end.

Bulk tank samples were processed by inoculating 50 µL of undiluted milk and 50 µL of a 10^{-1} dilution on 4 media types: Trypticase Soy Agar with 0.5% sheeps' blood and 0.1% esculing (Hardy Diagnostics, Santa Maria, CA), MacConkey media, Vogel-Johnson media and Edwards media (Northeast Laboratory, Belin, CT). Ten grams of bedding material was weighted out and agitated in 90 mLs of phosphate buffered solution in a stomacher and then diluted out to a concentration of 10^{-5} (5 dilutions). Fifty microliters of each dilution was plated on to the same 4 media types. Swabs were vortexed in Todd Hewett broth (Hardy Diagnostics, Santa Maria, CA) and then approximately 10µL was plated on the 4 types of media. The remaining Todd Hewett broth was incubated overnight with 1.5 mL of M17 media broth (Becton, Dickinson and Company, Franklin Lakes, NJ) and the resulting combination was plated on to Edwards media. One half

millileter of bulk tank milk and the original agitated bedding liquid were also incubated with M17 broth and plated on Edwards media. All plates were incubated for 48 hours. Colony counts were performed on a plate with a dilution which resulted in <250 colonies per plate. A representative GPCN colony from the samples incubated in M17 broth was isolated on a new plate and identified using Matrix Assisted Laser Desorption Ionization Time-of-Flight technology (**MALDI-TOF**, Bruker Daltonics, Billerica, MA) at QMPS in Warsaw, NY.

Data was recorded in Microscoft Excel (Microsoft Corp., Santa Rosa, CA, 2010). Bacteria counts were transposed into logrythmic values for averaging and then transposed back for descriptive statistics. Dicotomous data was compared using chi square analysis.

Results:

Demographics

A total of 85 bedding samples, 20 bulk tank samples, and 900 swabs were taken from 20 farms over a 6-month period. Herd size ranged from 120 cows to 2,400 cows. One farm was milking in a tie stall barn (with some cows housed in free stall barns), but the remaining farms all had cows housed in free stall barns and milked in parlors. Parlor types included a step-up parlor with 7 units, a 12-unit swing parlor, 10- to 32-unit herringbone parlors (4 farms), and 16- to 80-unit parallel parlors (13 farms).

Bedding types included manure solids (3 farms), sand (12 farms), and other organic material (2 sawdust, 2 chopped hay/straw and 1 Syracuse Fiber). Several of the farms used other bedding materials, but analysis was done based on bedding used in the fresh pen.

Further information on demographsics can be seen in Appendix: Table 1. All farms wore gloves during milking and used pre and post dip routinely. One farm does not forestrip, and two farms do not use individual towels to clean teats (one towel is used for 2 cows).

Bulk Tanks

Average bacteria counts for all types of bacteria are shown in Appendix: Figure 1, comparing the entire population of bulk tanks, LP herds and LN herds.

The number of GPCN bacteria found ranged from 220 cfu/mL to >50,000 cfu/mL across all samples. The average for the entire population was 1,600 cfu/ml (n=20), 2,093 cfu/ml in LP herds (>1% *Lactococcus* spp in CM samples) (n=10), and 1,222 cfu/mL in LN herds (≤1% *Lactococcus* spp in CM samples) (n=10). There was no difference between the two groups and no correlation between the number of GPCN organisms identified in bulk tanks and the level of *Lactococcus* in the CM cases in the herd (P>0.1). Two bulk tank samples had *Lactococcus* spp. identified in the sample, and both of those farms were known to be LP herds. However, the small sample size in this preliminary trial prevents further interpretation on the accuracy of utilizing a bulk tank sample to screen for *Lactococcus* spp. infections in a herd. This aspect of the investigation was funded through the New York Farm Viability Institute and will be compiled with data

from herds across the state of New York. That dataset will yield very valuable information as it is analyzed.

Bedding

Eighty five bedding samples were collected from all 20 farms, including 5 types of bedding:

- sand (n= 32 from LP herds, 12 from LN herds),
- sawdust (12 LN herds),
- manure solids (n=4 LP herds, 9 LN herds),
- chopped hay/straw (n=8 LN herds), and
- Syracuse Fiber (n=4 LP herds, 4 LN herds).

Average number of cfu/g of bedding can be see in Appendix: Figure 2. The average bacteria counts of GPCN were higher in used bedding compared to new bedding, with manure solids having the highest number of GPCN organisms in used samples.

Used samples from LP herds had a higher number of GPCN organisms when compared to LN herds.

Differences between levels of GPCN in different types of bedding based on *Lactococcus* spp. infection status of the herd was not yet fully assessed due to small sample size for each bedding type in this preliminary investigation. This aspect of the research was supported through a New York Farm Viability grant which allowed for sampling of bedding from 20 farms in the region. This information will be compiled with information from about 100 farms across the state, yielding more powerful results.

Lactococcus spp. was positively identified with MALDI-TOF in 8 bedding samples, all of which were sand, 2 were new sand samples and 6 were used sand sampled. Two *Lactococcus* spp. isolates originated from LN herds (1 new and 1 used) and 6 were from LP herds (2 new and 4 used).

Teat Swabs

Nine hundred teat swabs were collected from 300 cows across the 20 farms. Each cow was swabbed at three time points. Teat swabs plated on Edwards media were assessed after incubation for level of GPCN bacteria present: not detected (no growth), low (<20 cfu/plate), medium (20-50 cfu/plate) or high (>50 cfu/plate). The percentage of teats which were categorized at each level can be seen in Appendix: Figure 3 and compared by *Lactococcus* spp. infection status. The level of GPCN organisms was not higher on teat swabs from cows from LP herds.

Bacterial Isolates

From the 900 teat swabs, 20 bulk tanks and 85 bedding sample cultures collected, 880 samples had growth for analysis from 984 individual colonies that were identified to the genus and species level using MALDI-TOF (19 tanks, 71 bedding, and 790 swabs). Up to three unique colony types were submitted per sample. Samples that had no growth or were contaminated yielded no isolates for MALDI-TOF identification.

A total of 56 organisms were identified, and the organisms identified ten or more times are listed in Appendix: Table 2. Less common organisms of the genus *Streptococcus* and *Enterococcus* are grouped. *Aerococcus viridans* was the most common organism isolated from 465 colonies. The next most common pathogen was *Lactococcus garvieae* from 113 colonies, and all other oganisms were identified fewer than 100 times.

The 130 colonies of *Lactococcus* spp. originated from 114 individual samples, including 2 tanks, 8 bedding samples (2 new, 6 used) and 104 teat swabs (62 pre-milking, 21 post-prep and 21 post-milking). When differentiated by *Lactococcus* spp. infection status, 87 isolates were identified on LP farms while 27 were identified on LN farms.

When looking at the organisms isolated from teat swabs, *Lactococcus* spp. was identified in 79 swabs from LP herds (n=370) and 25 from LN herds (n=420, $P<0.05$).

In bedding samples, *Lactococcus* spp. was only found in samples from farms with sand (13 from LN herds, 87 from LP herds), sawdust (n=9 from LN herds) and manure solids (n=5 from LN herds) used as bedding types. Further breakdown of where *Lactococcus* spp. was identified can be seen in Appendix: Table 3.

Conclusions/Outcomes/Impacts:

While the true risk factors and causes of *Lactococcus* spp. mastitis are still being investigated, it does appear that cows on farms with >1% of CM caused by this organism are more likely to have it identified on teats. *Lactococcus* spp. does also appear more frequently on teats before pre-dipping, but can still be identified after teat disinfection.

The level of GPCN organisms on teat skin does not seem to be related to *Lactococcus* spp. IMIs. Bacteria levels of GPCN organisms in bedding were not able to be fully compared due to smaller sample sizes of organic bedding materials. Identification of *Lactococcus* spp. in known LP herds was difficult due to the presence of other GPCN organisms which could not be differentiated morphologically for further MALDI-TOF testing, and only was found in 2 tank samples.

This study was able to identify that isolation of *Lactococcus* spp. from environmental samples is difficult, requiring encouragement of the growth of these organisms through incubation in M17 broth. This is likely an economically prohibitive step in routine bulk tank cultures as a surveillance tool for *Lactococcus* spp. presence in a herd.

Lactococcus spp. is difficult to differentiate morphologically from other GPCN organisms, and due to the high prevalence of non-pathogenic GPCN organisms, environmental sampling for *Lactococcus* spp. is difficult and more elaborate testing may be necessary to fully evaluate bedding and bulk tank samples for this organism for on-farm application. Further data analysis and additional testing of the microbiomes of these samples may yield additional information to help dairy farmers reduce the risk of *Lactococcus* spp. mastitis in their herds.

As the behavior of the *Lactococcus* spp category of IMIs is further investigated, this research does support the potential that *Lactococcus* spp. are environmental organisms and may be more prevalent on some farms. Farms should focus on ensuring that milking occurs on clean, dry, well stimulated teats, that milking equipment is in good working order and that stalls are clean and dry.

For farms with a higher number of CM cows infected with *Lactococcus* spp., control may also involve segregation and milking infected animals last as it is still unknown if transmission through shedding of the organism into the milking unit poses a threat to the next cow milked.

Outreach:

Information on *Lactococcus* spp mastitis was presented at the following meetings:

- American Association of Bovine Practitioners research summaries, Charlotte, NC, Sept. 2016
- NYS Agriculture and Markets Certified Milk Inspector regional meetings, E. Aurora, Canton, Syracuse and Albany, NY, Sept. 2016
- Northeast Organic Dairy Producers Association, Chambersburg, PA, Sept. 2016
- QMPS Udder Health Workshop, Ithaca, NY, Oct. 2016
- CCE Milk Quality seminars, Lowville and Malone, NY, January 2017
- National Masitis Council Annual Meeting poster session, St. Petersburg, FL, Jan. 2017

Next Steps:

Bedding and bulk tank samples from this project will be analyzed for their unique microbiomes to help expand the information and knowledge base about this organism in environmental samples. In addition, the samples from cows with *Lactococcus* spp. gathered through the research funded by the farmer-driven NNYADP in 2014, 2015 and 2016 will undergo DNA sequencing in order to compare *Lactococcus* spp strains within and between farms, which will hopefully help indicate if there is an environmental versus contagious nature of this infection.

Acknowledgments:

This project would not have been possible without the assistance of the twenty collaborating dairy farms in Northern NY who allowed us to take environmental samples from their farms and cows. A special thanks to the staff of Quality Milk Production Services in Canton, NY, along with our summer preceptor Melissa Marlowe.

Reports and/or articles in which results of this project have been published:

- American Association of Bovine Practitioners conference proceedings 2016
- National Mastitis Council proceedings 2017
- "Lactococcus: an emerging mastitis pathogen." Dairybusiness & Holstein World. October 2016

For More Information:

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Northern NY Agricultural Development Program
2015-2016 Project Report APPENDIX

Speciation and Quantification of Streptococcus-like Bacteria from Bulk Tank Milk, Bedding, and Teat Swabs Pre- and Post-milking from Dairy Herds in Northern New York

Table 1: Demographics of 20 farms from which environmental samples were taken, separated based on level of infection by Lactococcus spp. in cows with clinical mastitis (>1% Lactococcus spp infections in CM: LP Herds, versus those with ≤1% Lactococcus spp infections in CM: LN Herds), NNYADP project, 2016.

Farms based on Lactococcus spp. infection status							
LN Herds[1]				LP Herds[2]			
Farm	**Herd size**	**bedding type**	**% Lactococcus**	**Farm**	**Herd size**	**bedding type**	**% Lactococcus**
B*	3000	manure solids	0.3%	K[¥]	2400	manure solids	4.3%
G	1200	sawdust	0.0%	S	1500	sand	1.6%
E	1100	mixed	0.0%	R	1200	sand	4.5%
A	830	manure solids	0.0%	T	1200	syr fiber	1.5%
D	800	sand	1.0%	N	1130	sand	2.6%
C*	800	mixed	0.0%	L	800	mixed	1.7%
H	700	sawdust	0.3%	O	800	sand	18.9%
F	400	sand	1.0%	Q	800	sand	20.6%
I	350	straw/chopped hay	0.0%	P	700	sand	8.1%
J	120	straw/chopped hay	0.0%	M	530	sand	18.9%

[1] "Without" farms have ≤1% Lactococcus sp identified in clinical mastitis samples
[2] "With" farms have >1% Lactococcus sp identified in clinical mastitis samples
[¥] indicates farms that do not forestrip
* indicates farms that use a single towel to wipe more than one cow.

Table 2. Most common organisms identified from environmental samples separated out by source of sample, NNYADP project, 2016. Organisms identified fewer than 10 times were not included. A total of 56 organisms were identified from 20 tank samples, 85 bedding samples and 900 teat swabs. Up to three morphologically different organisms were tested from each sample.

	Number found per source			
Organism	**Bedding**	**Swab**	**Tank**	**Total**
Aerococcus viridans	37	425	3	465
Lactococcus garvieae	8	104	1	113
Vagococcus fluvialis	5	60	1	66
Enterococcus faecalis	6	29	15	50
Streptococcus uberis	4	23	5	32
Enterococcus hirae	2	29	0	31
Enterococcus aquimarinus	0	29	0	29
Enterococcus pseudoavium	0	18	0	18
Enterococcus saccharolyticus	4	13	1	18
Lactococcus lactis	0	14	3	17
Entercoccus pseudoavium	0	16	0	16
Enterococcus casseliflavus	3	9	0	12
Enterococcus gallinarium	3	7	0	10
other *Streptococcus*	2	15	0	17
other *Enterococcus*	4	21	0	25

Table 3: Number of environmental and bulk tank samples with gram positive, catalase negative (GCPN) cocci to identify via Matrix Assisted Laser Desorption Ionization Time-of-Flight and number of samples where Lactococcus spp. were identified in herds with >1% Lactococcus spp infections in CM: LP Herds, versus those with ≤1% Lactococcus spp infections in CM: LN Herds, NNYADP project, 2016.

Environmenal Sample			Total submitted for MALDI-TOF identification		Total *Lactococcus* spp. found via MALDI-TOF		
			LN Herds	LP Herds	LH Herds	LP Herds	TOTAL
Bulk tank			**9**	**10**	**0**	**2**	**2**
Bedding*	Sand	new	5	5	1	2	3
		used	11	23	1	4	5
	Sawdust	new	2	-	-	-	-
		used	6	-	-	-	-
	Manure Solids	new	-	1	-	-	-
		used	4	3	-	-	-
	Syracuse Fiber	new	-	1	-	-	-
		used	-	3	-	-	-
	Hay/Straw	new	1	-	-	-	-
		used	6	-	-	-	-
	SUBTOTAL		**35**	**36**	**2**	**6**	**8**
Swabs	Pre prep		144	138	15	47	62
	Post prep		140	119	4	17	21
	Post milking		136	113	6	15	21
	SUBTOTAL		**420**	**370**	**25[a]**	**79[b]**	**104**
TOTAL			**464**	**416**	**27**	**87**	**114**

[ab]indicates a difference in number of samples where *Lactococcus* spp. was identified using chi square analysis ($P<0.05$).

Figure 1: Average bacteria counts (cfu/ml) for all bulk tanks, bulk tanks from herds with >1% Lactococcus spp infections in CM: LP Herds, and bulk tanks with ≤1% Lactococcus spp infections in CM: LN Herds, NNYADP project, 2016.

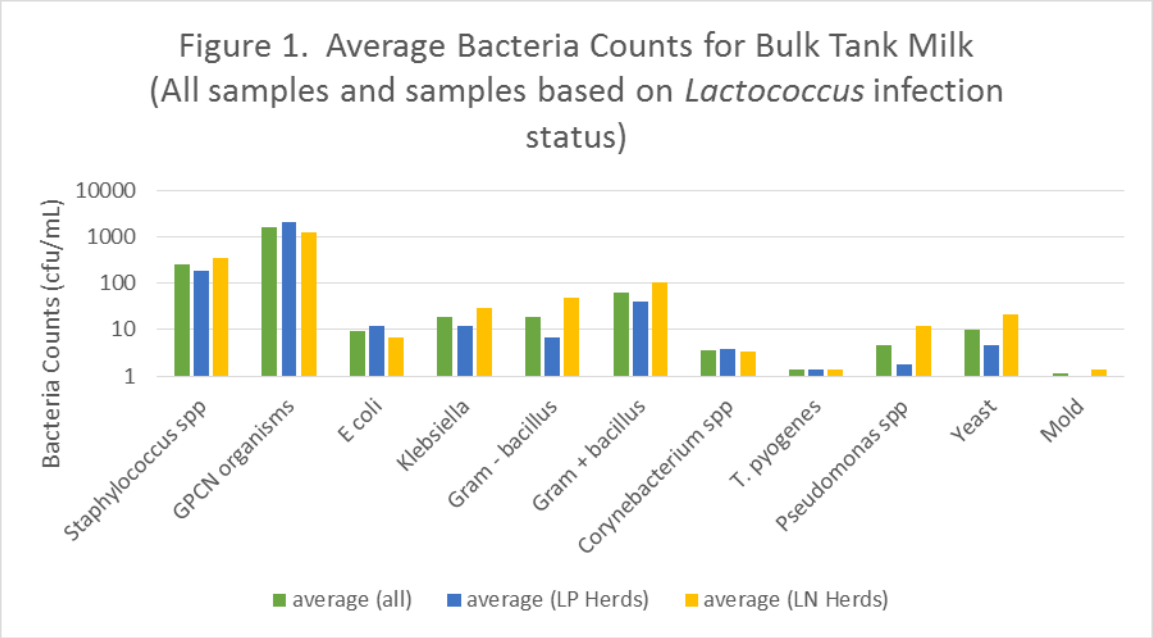
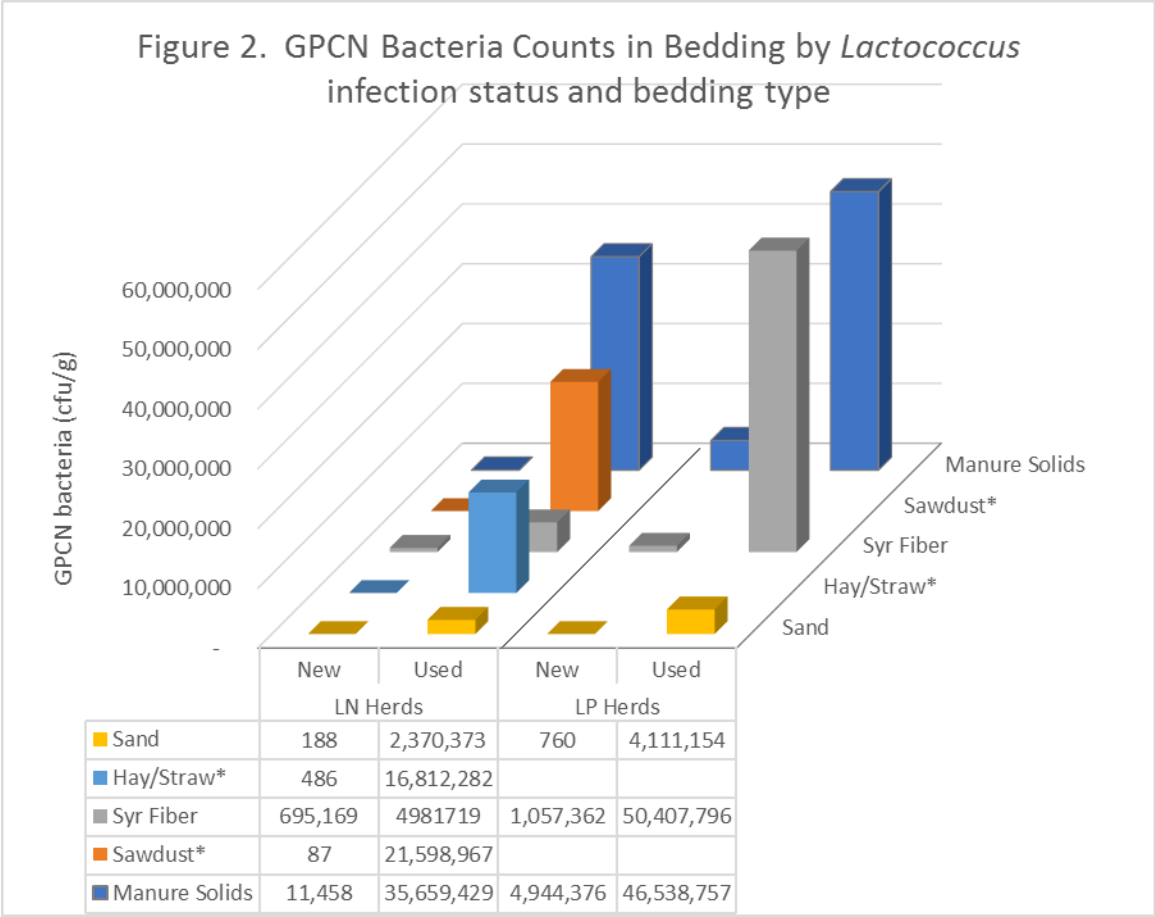


Figure 2: Levels of GPCN bacteria found in new (n=21) and used (n=64) fresh pen bedding samples from herds with >1% Lactococcus spp infections in CM (LP Herds) to those with ≤1% Lactococcus spp infections in CM (LN Herds). *There were no LP herds with hay/straw or sawdust as bedding sources in the fresh pen.



	LN Herds		LP Herds	
	New	Used	New	Used
Sand	188	2,370,373	760	4,111,154
Hay/Straw*	486	16,812,282		
Syr Fiber	695,169	4981719	1,057,362	50,407,796
Sawdust*	87	21,598,967		
Manure Solids	11,458	35,659,429	4,944,376	46,538,757

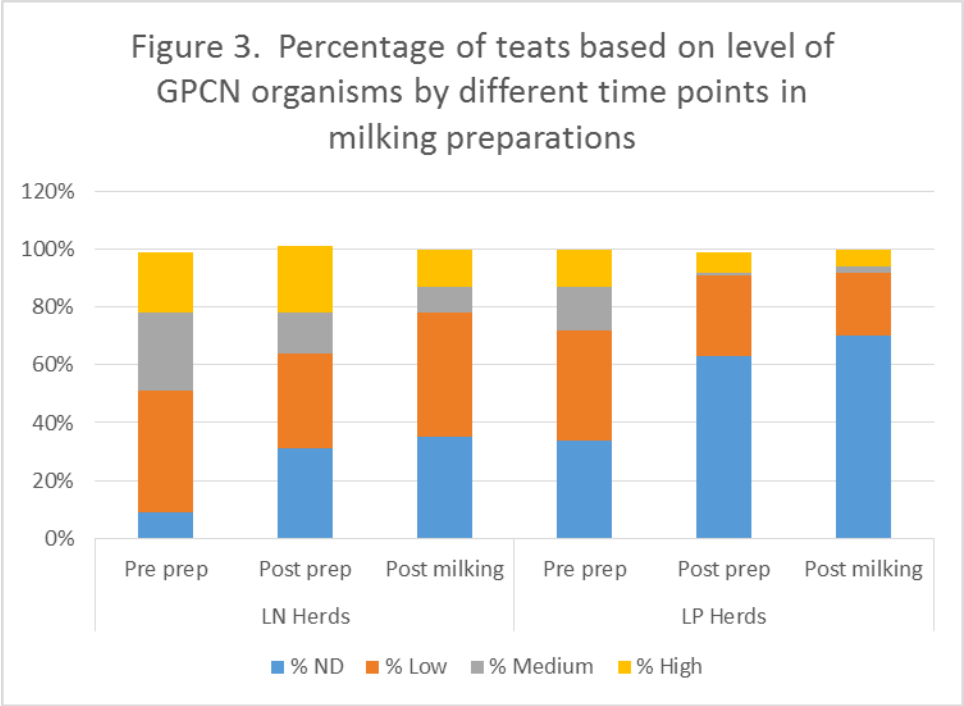


Figure 3: Percentage of teats based on level of gram positive, catalase negative (GCPN) organisms from teat skin swabs at three different time points, comparing cows from herds with >1% Lactococcus spp infections in CM: LP Herds, to those with ≤1% Lactococcus spp infections in CM: LN Herds, NNYADP project, 2016. Level of bacteria was classified as not detected (ND, no growth), low (<20 cfu/plate), medium (20-50 cfu/plate), or high (>50 cfu/plate) based on bacterial growth on Edwards media.