Evaluation of selective dry cow therapy for controlling mastitis and improving antibiotic stewardship in U.S. dairy herds

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1 CHAPTER ONE: LITERATURE REVIEW

1.1 Intramammary Infection and Mastitis

Mastitis is the most important infectious disease of dairy cows, impairing cow health, production and welfare, as well as threatening profitability of dairy farms. From a pathophysiological standpoint, mastitis is defined as inflammation of the mammary gland, which often occurs following intramammary infection (**IMI**) by pathogenic micro-organisms (Watts, 1988). In most cases, the route of infection is by retrograde passage through the teat canal. However, hematogenous spread to the mammary gland has also been suggested as potential route of infection for some pathogens (Fox et al., 2005). The presence of pathogens in the mammary gland can provoke an inflammatory response from the cow's immune system, which in combination with pathogen virulence factors (such as exotoxins) can harm the cow and reduce her ability to produce milk that is fit for human consumption.

Clinical mastitis is typically characterized by the presence of abnormal milk; usually clotted, watery and/or discolored. Other clinical signs include pyrexia and inappetence, swelling, heat and painfulness of the udder. Cows with peracute clinical mastitis can become recumbent and die within hours of onset of clinical signs. Cows can also be affected by subclinical mastitis, which is characterized by grossly normal milk, with elevations of inflammatory indicators in milk like somatic cell counts (**SCC**).

1.2 Impact of Mastitis

Mastitis is recognized by farmers and the dairy industry as an important source of reduced welfare for dairy cows (Ventura et al., 2015). These concerns tend to be focused primarily on the pain and culling associated with clinical mastitis. Furthermore, a single case of clinical mastitis is estimated to cost the producer $444 USD (Rollin et al., 2015). This accounts for increased expenditure for therapeutics, diagnostics, labor and purchasing replacement animals, in addition to losses from reduced future milk production and culling or death. Consequently, given that up to 50% of cows will experience at least one case per lactation (Erskine et al., 1988, Riekerink et al., 2008), the financial impact of mastitis surpasses any other disease experienced on dairy farms (Kaneene and Scott Hurd, 1990, Miller and Dorn, 1990, Kossaibati and Esslemont, 1997).

Mastitis treatment and prevention also contributes a significant proportion of total antibiotic use in adult dairy cattle in the U.S. (66%), Belgium (64%) and New Zealand (43%) (Pol and Ruegg, 2007, Stevens et al., 2016, Bryan and Hea, 2017). Dry cow therapy (DCT), which is the use of long-acting, high concentration antibiotics at dry-off for mastitis control, accounted for 29 to 35% of total antibiotic use in these studies. Consequently, reducing the incidence of mastitis on dairy farms will improve cow health and welfare, increase financial returns for producers and improve antibiotic stewardship within the dairy industry.

1.3 Mastitis Pathogens

Endemic mastitis pathogens can establish reservoirs in the mammary glands of infected cows, the environment, or in both sites concurrently. The pathogens with a predilection for environmental reservoirs (aka "environmental pathogens") include coliform bacteria, like *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp., as well as *Streptococcus* spp. other than *Streptococcus agalactiae*, and Strep-like organisms, like *Lactococcus* spp. and *Enterococcus* spp. It has also been suggested some non-aureus *Staphylococcus* spp. (NAS) may establish reservoirs in the environment (Piessens et al., 2011). Cows become exposed to environmental pathogens as a result of teat contamination with bedding material, manure, and dirt. Pathogens that typically establish reservoirs in the mammary glands of infected cows (aka "contagious pathogens") include *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*. Cows become exposed to these pathogens when teats are contaminated with milk from infected cows, often via fomites such as the hands of milking staff, udder towels and teat-cup liners (Fox et al., 1991, Zadoks et al., 2002). Implementation of mastitis control strategies, like post-milking teat disinfection, DCT, milking machine maintenance and culling of chronically infected cows (Neave et al., 1966) has resulted in a reduction in the prevalence of these pathogens in dairy herds of developed countries (Ruegg, 2017), such that environmental pathogens are now the predominant cause of clinical mastitis (Riekerink et al., 2008).

Mastitis pathogens are often grouped as being 'major', 'minor' or neither, with major pathogens being those that are commonly associated with clinical mastitis. The following pathogens are often considered to be 'major' in research studies: *Staphylococcus aureus,*

Streptococcus spp., and Strep-like organisms *(i.e. Aerococcus* spp., *Enterococcus* spp*., Lactococcus* spp., *Streptococcus* spp.), coliforms, *Trueperella pyogenes,* and yeasts. (Gohary and McDougall, 2018, Lipkens et al., 2019). Minor pathogens include non-aureus *Staphylococcus* spp., *Corynebacterium* spp. and in some studies, *Bacillus* spp. Other pathogens such as *Prototheca* spp. are often not considered to be major or minor pathogens.

1.4 Intramammary Infection during the Dry Period

Infection rates during the dry period are higher than any other phases of the lactation cycle, especially around dry-off and calving (Bradley and Green, 2004). This is due to an increased risk of exposure in many herds during this time and a decrease in resistive capacity in the early and late dry period. Exposure typically occurs due to inadequate environmental management in calving areas, dry-cow pens and paddocks (Compton et al., 2007). Dry-period factors that reduce the capacity of the teat and mammary gland to resist infection include udder involution and colostrogenesis (resulting in physiological stress), reduced removal of pathogens from the gland that would usually occur with regular milking and delayed formation of a keratin plug in the teat canal (Hogan and Smith, 2003, Dingwell et al., 2004).

1.5 Impact of Intramammary Infections during the Dry Period

The impact of IMI established during the dry period, or IMI that persist from the previous lactation through the dry period has been investigated in longitudinal studies. Smith et al. (1985) found that approximately half of *Strep. uberis* and two thirds of coliform

infections during the dry period manifested as clinical mastitis during lactation, all of which occurred within 76 days of calving. In that study, and another by Bradley and Green (2000) approximately half of the clinical cases occurring in the first 76-100 days of lactation were due to IMI established during the dry period. Other studies have also demonstrated strong associations between IMI detected in early lactation and early lactation clinical mastitis. For example Green et al. (2002) found that quarters infected with *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Staphylococcus aureus* at calving were much more likely (odds ratios in excess of 200) to have clinical mastitis cases from which those pathogens were also isolated. Consequently, the cumulative evidence of studies discussed above, dry-period intervention studies (reviewed in section **Error! Reference source not found.**) and clinical experience has led to a consensus within much of the mastitis research community that control of IMI during the dry period is critical for reducing IMI prevalence at calving and consequently, preventing clinical and subclinical mastitis incidence during early lactation.

1.6 Epidemiology of Intramammary Infections during the Dry Period

There are three components of IMI dynamics that are likely to influence post-calving udder health: 1) risk of IMI at dry-off, 2) the risk of those IMI curing during the dry period and 3) the risk of uninfected quarters acquiring new IMI during the dry period. No large-scale surveys have been conducted in the U.S. to describe the epidemiology of dry period IMI dynamics. Small surveys have estimated the prevalence of IMI at dry-off to be 12.8% (Pantoja et al., 2009), 19.2% (Arruda et al., 2013) and 34.7% (Johnson et al., 2016). Similar sized studies have found prevalences of IMI at dry-off in regions outside

of the U.S. to be (in order of references) Canada (16.0%), South Africa (29.8%), England (71.7%), Europe (12.3%), New Zealand (40.5%) and Belgium (42.0%) (Sanford et al., 2006, Petzer et al., 2009, Bradley et al., 2011, Bradley et al., 2015, Gohary and McDougall, 2018, Lipkens et al., 2019). In all of those studies, the predominant pathogen group was NAS, which was isolated from more than 50% of infected quarters. The impact of NAS IMI on post-calving udder health is uncertain, as they have been shown to have a high spontaneous dry period cure risk (Vasquez et al., 2018) and often have minimal effects on clinical mastitis risk (Green et al., 2002) or milk production in the subsequent lactation (Vanderhaeghen et al., 2014). Although coliform IMI are important causes of dry period new IMI, their prevalence at dry-off and at calving is usually low (Smith et al., 1985, Bradley et al., 2015). For example, surveys of IMI at dry-off have reported quarter-level coliform prevalences of 0.9 (Hogan et al., 1989), 1.27 (Bradley et al., 2015), 2.2 (Arruda et al., 2013) and 2.7% (Johnson et al., 2016).

Dry period IMI cure risks in recent studies have been 90% (Ospina et al., 2016), 89% (Arruda et al., 2013), 85% (Johnson et al., 2016) and 80% (Bradley et al., 2010). Cure risks will depend on the pathogen causing the IMI, the chronicity of infection and in some cases, the therapeutic approach taken at dry-off. Dry period new IMI risk in recent studies were 18.5% (Johnson et al., 2016), 13.3% (Arruda et al., 2013) and 30% (Bradley et al., 2010). Factors impacting dry period IMI cure risk and new IMI risk are discussed later in this review. In summary, although a number of small studies have reported IMI prevalence and dynamics over the dry period, there have been no large, multi-site

observational studies conducted. Therefore more research is needed to understand the prevalence of IMI in late lactation cows in U.S. dairy herds.

1.7 Control of Dry Period Intramammary Infections

Udder health in the subsequent lactation can be optimized by reducing the prevalence of IMI among cows entering the dry period, maximizing the probability that IMI present at dry-off will cure during the dry period and minimizing the probability of quarters becoming infected during the dry period. Reducing the prevalence of IMI at dry-off is hypothesized to improve udder health in the subsequent lactation and reduce antibiotic use in a selective DCT (SDCT) program. The former hypothesis is supported by observational studies that have shown positive associations between IMI caused by major pathogens at dry-off and post-calving udder health (Østerås et al., 1999, Green et al., 2002, Newman et al., 2010). However, no intervention studies have shown a clear relationship between reducing IMI prevalence at dry-off and post-calving udder health. First principles would suggest that reducing the rate of new IMI during late lactation would subsequently reduce the probability that cows or quarters would test positive at dry-off in a SDCT program, and therefore reduce probability of antibiotic treatment.

A number of cow- and quarter-level interventions have been shown to impact dry period IMI cure and new IMI risk. Of these, the use of DCT and internal teat sealants (ITS) at dry-off are the most commonly studied therapeutic approaches, and are reviewed in subsequent sections of this chapter. However, it should be mentioned that other factors that are also likely to impact dry period cures include: age of cow, cause and chronicity

of IMI and dry period length (Østerås et al., 1999, Dingwell et al., 2002, van Hoeij et al., 2016). Factors that are likely to impact dry period new IMI risk include: milk yield at dry-off, cessation of milking approach (i.e. gradual verse abrupt), teat end hyperkeratosis, level of hygiene when administering DCT and environmental hygiene during the early and late dry periods (Dingwell et al., 2004, Rajala-Schultz et al., 2005, Compton et al., 2007, Green et al., 2007, Tucker et al., 2009, Newman et al., 2010, Gott et al., 2016).

1.7.1 Bedding Management and Intramammary Infection at Dry-Off

One potential source of exposure to IMI-causing bacteria is bedding material. This is supported by molecular epidemiologic studies that have identified IMI-causing strains of bacteria in bedding material, suggesting that bedding can act as a reservoir for some pathogens (Verbist et al., 2011, Eraclio et al., 2018). Consequently, producers and milk quality advisors use aerobic culture of bedding to determine bedding bacteria count (BBC) to approximate bedding-associated mastitis risk (Hogan et al., 1989). However, few studies have demonstrated a clear association between BBC and udder health, and none have evaluated the association between BBC and IMI in late lactation cows. Hogan et al. (1989) reported a positive association between counts of Gram-negative bacteria and *Klebsiella* spp. in bedding and clinical mastitis incidence in nine U.S. dairy herds. However, no associations were found with other pathogens, including environmental *Streptococcus spp*., which were a common cause of clinical mastitis in that study. Thomas et al. (1983) identified a positive association between BBC and clinical mastitis incidence for *Klebsiella spp*. but not *E. coli*. Similarly, a longitudinal study of herds using manure solids bedding found that when *Klebsiella spp.* counts exceeded 6.0 $\log_{10}$ CFU / g, the proportion of clinical cases caused by *Klebsiella spp.* increased (Carroll and Jasper,

1978). Natzke and LeClair (1976) failed to increase the incidence of new IMI in a small sample of cows, after inoculating their sawdust bedding with *E. coli* for four weeks. A recent cross-sectional study of 168 herds from 17 U.S. states found that counts of coliforms, *Klebsiella* spp., SSLO, and *Staphylococcus* spp. in commonly used bedding types were positively associated with clinical mastitis incidence and monthly SCC dynamics (Patel et al., 2019). However, the relationships varied among bedding material types for each udder health outcome. Furthermore, this study did not evaluate IMI, and enrolled cows from all stages of lactation.

There is also indirect evidence to suggest that high levels of bacteria in bedding can negatively affect udder health. Many studies have demonstrated a correlation between BBC and teat end bacteria count (Hogan and Smith, 1997, Hogan et al., 1999, Zdanowicz et al., 2004, Proietto et al., 2013, Rowbotham and Ruegg, 2016b). Extrapolating these associations to udder health is problematic, as no studies to our knowledge have demonstrated a strong association between teat end bacteria counts and mastitis. Consequently, there is currently a lack of rigorous evidence to support the widely held belief that high BBC is a risk factor for IMI and mastitis.

One pragmatic question facing dairy producers is the decision around bedding material choice, with the common options including recycled manure solids (**MS**), new sand (**NS**), recycled sand (**RS**) and organic non-manure (**ON**) bedding. It is generally expected that farms using bedding material types with higher BBC, such as MS, will have a higher prevalence of IMI or a different profile of pathogens causing IMI. Furthermore, certain

bedding material types may exhibit synergism with BBC in the development of IMI. These hypotheses are based on findings from observational studies where bedding bacterial populations and udder health outcomes varied according to bedding material type (Hogan et al., 1989, Zdanowicz et al., 2004, Bey et al., 2009, Rowbotham and Ruegg, 2015, 2016a, b, Patel et al., 2019). For example, a prospective cohort study reported by Rowbotham and Ruegg (2016b) found that NS bedding had the lowest counts of *Streptococcus* and Strep-like organisms (**SSLO**), Gram-negative and coliform bacteria, when compared with RS and MS. In the same study, primiparous cows exposed to NS had significantly lower teat end coliform counts and a tendency for a lower incidence rate of clinical mastitis than cows exposed to other bedding materials (Rowbotham and Ruegg, 2016a). A cross-sectional study of 325 Wisconsin dairy herds by the same research group also found that herds using sand bedding had lower somatic cell counts (**SCC**) and higher milk production than herds using organic bedding materials (Rowbotham and Ruegg, 2015). A recent cross-sectional study by Patel et al. (2019) found that herds using MS bedding had high BBC, worse udder hygiene, increased counts of coliforms and SSLO in bulk tank milk and worse udder health than herds using ON, NS and RS. However, that study did not evaluate IMI at the cow or quarter-level. In summary, more research is needed to understand how bedding management and BBC is related to IMI prevalence at dry-off.

1.7.2 Internal Teat Sealants

Internal teat sealants reduce IMI prevalence in early lactation by reducing dry period new IMI. Internal teat sealants are typically composed of 60 to 65% bismuth subnitrate, which is infused into the teat cistern and teat canal of cows following the cow's last milking.

This non-antibiotic, inert paste forms a physical barrier within the teat that prevents pathogens from infecting the mammary gland. Clinical trials have shown that infusing a 60% bismuth subnitrate product (Orbeseal / Teatseal) at dry-off reduces new IMI over the dry period by 25% (RR = 0.75) when used alone, or with antibiotics (Rabiee and Lean, 2013). Furthermore, this product has also been shown to reduce SCC and clinical mastitis risk in early lactation (Godden et al., 2003, Rabiee and Lean, 2013, Golder et al., 2016).

1.8 Dry Cow Antibiotic Therapy

Dry cow antibiotic therapy is the administration of intramammary (IMM), high-concentration, and long acting antibiotics at the time of dry-off. This practice gained widespread implementation in the 1960s as part of the 'five point plan' in the UK (Neave et al., 1966) and numerous clinical trials have demonstrated its efficacy in increasing IMI cure risk and decreasing new IMI risk during the dry period. Antibiotics (antibiotic class shown in parentheses) used in DCT formulations registered in the U.S. include procaine penicillin (natural penicillin), cloxacillin (beta-lactamase resistant penicillin), cephapirin (1st generation cephalosporin), ceftiofur (3rd generation cephalosporin), dihydrostreptomycin (aminoglycoside), and novobiocin (aminocoumarin). The latter two antibiotics are each, separately included in combination formulations with procaine penicillin. Antibiotics that are registered for use outside of the U.S. are mostly from the same antibiotic families, with most being beta-lactams (penicillins and cephalosporins). It should be noted that parenterally administered DCT has also been studied, but is not routinely practiced (Soback et al., 1990).

A substantial number of negatively-controlled clinical trials have been conducted in the past 50 years to evaluate the efficacy of DCT for curing IMI and preventing new IMI, many of which (n = 33) were included in a 2008 meta-analysis conducted by Halasa and colleagues. The first study (Halasa et al., 2009b), evaluated the effect of DCT on dry period new IMI risk. They found that the effect of DCT on new IMI differed between pathogens. For example, risk of new IMI caused by *Streptococcus* sp. was reduced by 61% (RR = 0.39, 95% CI: 0.30, 0.51, 13 studies). A protective effect was also observed against *Staphylococcus* sp. IMI (RR = 0.62, 95% CI: 0.47, 0.83, 18 studies), but model diagnostics indicated that this effect may have been due to publication bias. Finally, results from studies that evaluated new IMI caused by coliforms (n = 7), found no protection (RR = 0.95, 0.81, 1.10). The apparently lack of efficacy against gram-negative pathogens may have been due to the use of narrow spectrum antibiotics in most studies. The second meta-analysis evaluated the efficacy of DCT to cure IMI at dry-off (Halasa et al., 2009a), which found that DCT increased cure risk by 78% (RR = 1.78, 95% CI: 1.51, 2.10, 14 studies), which was observed for *Streptococcus* sp. (RR = 1.86, 95% CI: 1.48, 2.35) and *Staphylococcus* sp. (RR = 1.65, 95% CI: 1.38, 1.96). Other pathogens, including coliforms were not evaluated. Neither of the studies by Halasa and colleagues reported more tangible measures of udder health, like clinical mastitis and somatic cell count in the subsequent lactation, likely due to variability between studies for case definitions and periods at risk. However, individual negatively controlled studies have shown that DCT can reduce post-calving clinical mastitis incidence and somatic cell counts in early lactation (Eberhart, 1986, Cummins and McCaskey, 1987).

An additional finding of the meta-analyses conducted by Halasa and colleagues was that antibiotic class did not impact dry period IMI dynamics. In that study, cloxacillin was found have similar cure risks (RR = 1.0, 95% CI: 0.96, 1.06, 6 studies) and new IMI risk (RR = 1.09, 95% CI: 0.94, 1.25, 6 studies) to other products. However, no third or fourth generation cephalosporin antibiotics (i.e. broad spectrum antibiotics that are commonly used in North American and Europe respectively) were evaluated in that meta-analysis. Recent multi-site, non-inferiority trials conducted in the U.S. have found similar udder health outcomes in cows dried-off with broad (ceftiofur) and narrow spectrum (cloxacillin, cephapirin and penicillin-dihydrostreptomycin) DCT products (Arruda et al., 2013, Johnson et al., 2016). However, some studies have shown differences in DCT product performance. One small trial conducted by Pinedo et al. (2012) in two Florida herds found that treatment with a narrow spectrum antibiotic (penicillin-dihydrostreptomycin) resulted in higher incidences of clinical and subclinical mastitis in the first 60 days after calving, when compared to treatment with a broad spectrum product (ceftiofur). A clinical trial conducted in England (Bradley et al., 2011) found that quarters treated with a 4th generation cephalosporin antibiotic (cefquinome) at dry-off had lower post-calving prevalence of IMI caused by major pathogens than cloxacillin (odds ratio = 0.62, 95% CI: 0.41, 0.93) and lower rate of clinical mastitis during the first 100 days of lactation (hazard ratio = 0.49, 95% CI: 0.25, 0.93). This improvement in udder health was attributed to a lower new IMI risk during the dry period (3.6% vs 6.1%). In the same study, a third treatment group received a combination of a narrow spectrum antibiotic (cloxacillin) and a commercial ITS, which was found to have similar udder health outcomes to the broad spectrum group, which was not concurrently treated with

ITS. In summary, the current evidence suggests that antibiotic product choice is unlikely to have a strong impact on post-calving udder health when ITS is concurrently used.

1.9 Selection of Cows to Receive Dry Cow Therapy

Given the growing expectation for producers to justify and reduce their antibiotic use where possible, there is increasing interest in improving the efficiency of antibiotic use at dry-off. There are two general approaches to DCT: blanket and selective. The most recent survey of U.S. dairy producers estimated that 80% currently practice blanket DCT, with 10% practicing selective DCT and 10% not using any DCT. Consequently, BDCT is considered to be the industry standard practice in dairy herds in North America, Australasia and in some European countries.

1.9.1 Blanket Dry Cow Therapy

Blanket dry cow therapy involves treatment of all quarters of all cows, irrespective of the infection status of the quarter. This practice was originally proposed as part of the five-point plan in the United Kingdom and was subsequently implemented in the U.S.A and remains the official recommendation of the National Mastitis Council. There are several advantages to practicing BDCT. Firstly, BDCT does not require farm staff to make individual decisions at the quarter- or cow-level, which facilitates consistent, accurate implementation of DCT protocols. Secondly, BDCT theoretically maximizes the herd-level therapeutic benefit of DCT, by ensuring that all cows that require treatment receive it. The widespread implementation of BDCT in past decades contributed to the decline in the prevalence of contagious mastitis pathogens like *Staphylococcus aureus* and

Streptococcus agalactiae, and to improvements in milk quality, which has been of great benefit to the dairy industry (Bradley, 2002, Ruegg, 2017). However, the quarter-level prevalence of IMI at dry-off in U.S. herds is likely to be less than 35%, with most IMI at dry-off being caused by minor pathogens (Pantoja et al., 2009, Arruda et al., 2013, Johnson et al., 2016), indicating that most quarters that receive DCT are not infected, and may not benefit from antibiotic treatment. Consequently, there is great interest to evaluate alternative DCT approaches.

1.9.2 Complete Cessation of Dry Cow Therapy

There are two alternatives to BDCT: complete cessation of DCT and SDCT. In 2014, the proportion of U.S. herds conducting SDCT and no DCT were both approximately 10% each (NAHMS, 2014b). The obvious advantage of removing DCT from the dry-off process is that it will drastically reduce overall antibiotic use. However decades of research has shown superior udder health outcomes in cows receiving DCT, when compared to negative controls (Eberhart, 1986, Cummins and McCaskey, 1987, Halasa et al., 2009a, Halasa et al., 2009b). Therefore, health could be negatively impacted if such a dry-off practice was implemented. However, it is important to note that most of those studies did not include the use of ITS, and were conducted > 20 years ago, when the prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* were much higher than they are now. Furthermore, the recent success of SDCT protocols in clinical trials raises the possibility that well-managed herds (low prevalence of IMI at dry-off and risk during the dry period) may not require DCT at all.

1.9.3 Selective Dry Cow Therapy

Selective DCT uses a screening strategy to determine which cows or quarters are likely to benefit from DCT and those that are not. Therefore, the ideal SDCT program delivers equivalent udder health to BDCT but with significantly less antibiotic use. Identification of IMI by direct tests, such as milk culture, is currently the ideal method for selecting quarters of cows to receive antibiotic dry cow therapy, as IMI at dry-off are associated with reduced udder health in the subsequent lactation (Green et al., 2002) and because demonstration of infection is a recommended prerequisite for antibiotic therapy in veterinary medicine (WHO, 2017). However, bacterial culture at a commercial reference laboratory is often impractical, time consuming and costly for producers. Consequently, most research has focused on tools that may be used on farm, including the use of rapid culture systems or predictive algorithms. Validated rapid culture systems for SDCT include 3M™ Petrifilm™ (3M Canada, London, ON, Canada) and the Minnesota Easy® 4Cast® plate (University of Minnesota, St Paul, MN). Under a culture-guided SDCT program, quarters or cows receive DCT if pathogens can be cultured from aseptic milk samples collected prior to dry-off. Algorithms typically use individual cow SCC and health records to identify candidate cows for DCT. Under a typical algorithm-guided SDCT program, a cow with a recent episode of clinical mastitis or elevated SCC are selected for DCT, as they are more likely to have an IMI than their herd-mates (Østerås et al., 1999). Herds will be unlikely to voluntarily adopt SDCT if it fails to deliver equivalent health outcomes to BDCT and substantial reductions in antibiotic use. Furthermore, SDCT protocols need to be practical and economical to implement. The subsequent sections of this chapter review the impact of various SDCT strategies on

health and antibiotic use, the accuracy and feasibility of screening tests used in SDCT and the economic impact of SDCT.

1.10 Impact of Selective Dry Cow Therapy on Antibiotic Use

Trials have found that SDCT can reduce quarter-level antibiotic use by 21 to 85% (Rajala-Schultz et al., 2011, Cameron et al., 2014, Scherpenzeel et al., 2014, Patel et al., 2017, Vasquez et al., 2018, McParland et al., 2019). Substantial reductions in antibiotic use at the industry-level has also been observed in European countries following nation-wide bans on BDCT (Vanhoudt et al., 2018). To date, no studies have compared antibiotic reductions for quarter-level culture-guided SDCT to algorithm-guided SDCT. However a recent study in Germany found that algorithm-guided SDCT (55%) reduced antibiotic use more than cow-level culture-guided SDCT (23%). No studies have specifically investigated factors that might drive antibiotic use in a SDCT program, however it is self-evident that high incidences of clinical and subclinical (elevations in SCC) mastitis during lactation will increase the risk of cows being classified as 'high risk' at the time of dry-off, and therefore eligible for antibiotic treatment in algorithm-guided SDCT. Furthermore, cows are more likely to be classified as high-risk when algorithms employ lower thresholds for SCC and/or clinical mastitis and incorporate longer periods of the lactation (eg. whole lactation vs last three tests vs last test). For example, the largest reduction in antibiotic use demonstrated was 85% in a clinical trial by Scherpenzeel et al. (2014), which only considered the last SCC test prior to dry-off and a relatively high SCC threshold in multiparous cows (> 250,000 cells/ml, >150,000 cells/ml for primiparous) to determine which cows received antibiotics. In contrast,

Cameron et al. (2014) reduced antibiotic use by only 21%, due to the use of lower SCC threshold in multiparous cows, and the use of additional tests. In that study, cows were eligible for antibiotic treatment if they met any of the following criteria: SCC > 200,000 cells/ml at any last 3 tests prior to dry-off; 1 or more cases of clinical mastitis during that same period; and bacterial growth from a pooled milk sample (all four quarters comingled in a single sample). Factors that will influence antibiotic use in culture-guided SDCT include prevalence of IMI, the culture of quarter samples vs composite samples (to make quarter- vs cow-level treatment decisions), contamination of milk samples and mistaken identification of microbial growth on the culture media. In summary, more research is needed to evaluate factors that are likely to impact antibiotic reductions so that SDCT programs can be optimized to reduce antibiotic use.

1.11 Impact of Selective Dry Cow Therapy on Health

An important barrier to the implementation of SDCT is the perception that it can negatively affect udder health. Such negative health outcomes can arise when the program fails to detect and treat IMI at dry-off (i.e. lower cure risk), or when untreated quarters are more vulnerable to new IMI during the dry period (higher new IMI risk). Consequently, most trials have focused on evaluating the effect of SDCT (compared to BDCT) on IMI dynamics during the dry period (IMI cure and new IMI risk) and IMI prevalence in early lactation. In addition, some studies have also reported more tangible and clinically relevant measures of udder health, including clinical mastitis risk, SCC and milk yield in the first 1-3 months of the subsequent lactation.

Of the 11 clinical trials evaluating culture- and algorithm-guided SDCT in the past 20 years, 3 found that SDCT impaired udder health (Berry and Hillerton, 2002, McDougall, 2010, Scherpenzeel et al., 2014), 3 had marginal impacts (Rajala-Schultz et al., 2011, tho Seeth et al., 2017, McParland et al., 2019), and 5 had negligible impacts (Bradley et al., 2010, Cameron et al., 2014, Cameron et al., 2015, Patel et al., 2017, Vasquez et al., 2018, Kabera et al., 2019). Subjective assessment of the existing research indicates that the use of ITS is a critical ingredient for SDCT success, as all trials with clear negative impacts did not use ITS, and all successful SDCT programs did use ITS. However, two recent studies using culture- and algorithm-guided SDCT with ITS showed slightly worse udder health outcomes than BDCT (tho Seeth et al., 2017, McParland et al., 2019). In both studies, *Staphylococcus aureus* were among the most common causes of IMI at dry-off.

Below is a discussion on SDCT trials recently conducted in North American herds (Rajala-Schultz et al., 2011, Cameron et al., 2014, Cameron et al., 2015, Patel et al., 2017, Vasquez et al., 2018, Kabera et al., 2019). The earliest study (Rajala-Schultz et al., 2011), evaluated the use of algorithm-guided SDCT in 4 herds in Ohio. Cows classified as low-risk by the algorithm (SCC < 200,000 cells at all tests in the 3 months prior to dry-off, no cases of clinical mastitis during the entire lactation) were randomized to receive DCT (n = 194, 500mg IMM benzathine cloxacillin) or no DCT (n = 192). Internal teat sealants were not used in either treatment group. Analysis found that the effect of SDCT on health and production outcomes varied between herds, indicating that herd-level factors may modify the efficacy of SDCT. When averaging across herds, the prevalence of IMI at calving was lower (48% vs 35%) in cows randomized to DCT, as were post-

calving SCC (-35,000 cells/ml). Milk yield and clinical mastitis incidences in the subsequent lactation were similar.

The next study to evaluate SDCT in North America was a multisite trial conducted in 16 commercial herds in Canada (Cameron et al., 2014, Cameron et al., 2015). Low risk cows (SCC < 200,000 cells/ml at the last 3 tests, no cases of clinical mastitis during the same time period) were recruited from low SCC herds and randomized to a BDCT (n = 369) or culture-guided SDCT (n = 360) group. Cows in the BDCT group received antibiotic (500mg ceftiofur hydrochloride IMM) and ITS treatment in all quarters. Cows in the culture-guided SDCT group were treated with DCT and ITS, if microbial growth was detected after 24hrs incubation of a cow-level composite milk sample on 3M™ Petrifilm™ (3M Canada, London, ON, Canada). If no growth was detected, then the cow received ITS in all four quarters. Therefore, investigators in this study used a combination of an algorithm and rapid culture for this SDCT program. Under this program, antibiotic use was predicted to be reduced by 21% if it was implemented across all cows in the study. Prevalence of IMI at calving and IMI cures during the dry period were similar between groups (Cameron et al., 2014). Furthermore, clinical mastitis rates and SCC in the subsequent lactation were similar between groups (Cameron et al., 2014, Cameron et al., 2015).

More recently, members of the same Canadian research group conducted a multi-herd (n = 9) clinical trial using culture-guided SDCT. In contrast to Cameron et al. (2014), all cows were eligible for inclusion (no algorithm was used), and diagnosis and treatment

was allocated at the quarter-level. This study has not yet been published in a peer-reviewed journal, but conference proceedings indicate that antibiotic use was reduced by approximately 55% and health outcomes were similar between treatment groups (Kabera et al., 2019).

Patel et al. (2017) conducted a pilot study evaluating the use of quarter-level culture-guided SDCT. In that study, cows from a university dairy herd in Minnesota were randomized to BDCT (n = 27) or culture-guided SDCT (n = 27), which used a rapid culture system (Minnesota Easy® 4Cast®, University of Minnesota, St Paul, MN) to allocate DCT (500mg ceftiofur hydrochloride IMM) at the quarter-level. All cows were treated with ITS. Antibiotic use was reduced by 48%, and IMI cures (82.3% vs 88.0%), new IMI (41.5% vs 40.2%) and IMI prevalence at calving (42.1% vs 39.6%) were similar between groups. The authors concluded that further research was needed to evaluate the use of culture-guided SDCT in U.S. dairy herds.

Finally, a clinical trial was recently conducted to evaluate algorithm-guided SDCT in a commercial dairy herd in New York (Vasquez et al., 2018). That study followed similar design to Rajala-Schultz et al. (2011), where low-risk cows (SCC < 200,000 cells/mL at last test, an average SCC < 200,000 cells/mL over the last 3 tests, <2 cases of clinical mastitis event in the current lactation) were randomized to a DCT (n = 304; 300mg benzathine cephapirin) and no DCT (n = 307). All cows received ITS. Antibiotic use was expected to be reduced by 60% if this approach was applied across the whole herd. Aside from slightly higher dry period IMI cure risk in the DCT group (93% vs 88%), IMI

dynamics were similar between groups. In summary, mixed results have been observed in trials evaluating the impact of SDCT on udder health and antibiotic use. Therefore, more research is needed to evaluate SDCT in U.S. dairy herds.

1.12 Performance of Screening Tests used in Selective Dry Cow Therapy

One critical component for any successful SDCT program will be the use of a diagnostic test with sufficient ability to identify cows or quarters likely to benefit from antibiotic therapy. A number of trials have evaluated the ability of screening tests to identify IMI in late lactation cows. Such studies report test characteristics like sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV) and apparent prevalence. In the context of SDCT, the SE and SP of a screening test indicates the probability of infected and uninfected quarters being treated and not-treated, respectively. Apparent prevalence and NPV are also useful test characteristics, as they provide an evaluation of the test performance at a given prevalence of disease. Specifically, the apparent prevalence gives an indication of the proportion of quarters that would receive antibiotic treatment under a given SDCT program, whereas the NPV indicates the risk of test-negative (i.e. untreated) quarters being uninfected. Thus, the NPV can be used to estimate the potential for negative udder health impacts caused by failing to treat a truly infected quarter. Interpretation of the test characteristics that serve as a proxy for udder health risks (SE / NPV) is somewhat subjective, as it is not clear what critical levels are required for a SDCT program to avoid negative udder health impacts.

Studies evaluating algorithms that use SCC and clinical mastitis history have consistently found poor agreement with the reference test (laboratory culture) when all pathogens are considered significant, with SE and SP never simultaneously exceeding 0.80 (Torres et al., 2008, Pantoja et al., 2009, McDougall and Compton, 2014, Kiesner et al., 2016, Gohary and McDougall, 2018). The predictive ability of algorithms improves when only IMI caused by major pathogens are considered (Pantoja et al., 2009, McDougall and Compton, 2014, Lipkens et al., 2019), which may be due to major pathogens being more likely than minor pathogens to cause clinical mastitis and marked increases in SCC (Honkanen-Buzalski and Bramley, 1984, Piccart et al., 2016). This apparent selection against minor pathogens may be an advantage of algorithm-guided SDCT as it is unclear if quarters infected with minor pathogens benefit from antibiotic treatment. However, this hypothesis requires investigation. Furthermore, some proponents of algorithm-guided SDCT suggest that the objective of algorithms is to identify cows with IMI and those at higher risk of developing new IMI than herd-mates (Vasquez et al., 2018). However this has not yet been clearly demonstrated. Studies have shown that shifting SCC thresholds induces a near-linear trade-off between SE and SP (Torres et al., 2008, Pantoja et al., 2009, Gohary and McDougall, 2018, Lipkens et al., 2019). Consequently, it is unlikely that a single SCC threshold will perform substantially better than others. Therefore, SCC thresholds should be selected by producers to match their aspirations for antibiotic reduction and their attitude toward risk for negative impacts on udder health.

Rapid culture methods have been evaluated in recent studies. Patel et al. (2017) evaluated the Minnesota Easy® 4Cast® plate in 103 quarters. They found that SE, SP, PPV and

NPV were 0.83, 0.73, 0.62, 0.89, when prevalence of IMI was 37%. Cameron et al. (2013) evaluated 3M™ Petrifilm™ in low-risk cows (SCC < 200,000 cells/ml at the last 3 tests, no cases of clinical mastitis during the same time period) at dry-off (n = 360 cows). In contrast to Patel et al. (2017), rapid-culture was conducted at the cow-level. Sensitivity, SP, PPV and NPV for 3M™ Petrifilm™ were found to be 0.85, 0.73, 0.71, and 0.87 when considering IMI at the cow-level. Therefore, the findings from these recent studies indicate that agreement between rapid culture and laboratory culture is higher than the agreement between algorithms and laboratory culture. This is unsurprising given that rapid culture uses very similar methodology to laboratory culture.

1.13 Practical Considerations for Culture- and Algorithm-Guided SDCT

For SDCT to be a widely accessible practice for commercial dairy producers, it must be practical. One likely reason for the widespread implementation of BDCT in commercial herds around the world is the simplicity of the procedure. Firstly, BDCT can be conducted quickly (approximately 3-5 minutes per cow) following the last milking, and the procedure is consistent across all cows in the herd. Selective DCT on the other hand, requires data to be collected so that decisions can be made at the cow- or quarter-level, which can potentially increase the time required for the dry-off procedure.

It is also important to contrast the practical considerations for culture- and algorithm-guided SDCT. The potential advantage of culture-guided SDCT is that it does not require herds to conduct monthly DHIA testing or record clinical mastitis during lactation, which currently accounts for approximately only 44% and 27% of herds in the U.S, respectively (NAHMS, 2014a). Furthermore, the use of rapid culture systems may enable farmers to

identify cows infected with pathogens of interest for specific interventions (eg. culling *Staphylococcus aureus* cows).

However, in a culture-guided SDCT approach, quarter-level detection and treatment is more complex than other DCT approaches, which may increase the risk of farm workers making mistakes during the dry-off procedure. Also, rapid culture requires that aseptic milk samples be collected at least two days prior to dry-off, which necessitates substantial allocation of labor and time. Finally, culture-guided SDCT requires that a laboratory be established either at the farm or at a local veterinary clinic for sample processing, plate incubation, and plate reading.

Predictive algorithms have significant advantages over other testing strategies. Firstly, algorithm-guided SDCT efficiently utilizes data that is already available on many farms, thus allowing the selection to be conducted automatically in advance of the dry-off event, which eliminates the time-lag between the commencement of the dry-off process and the administration of treatments. Furthermore, allocation of treatments at the cow-level is likely to be easier for farm staff to successfully implement than quarter-level treatments. The disadvantages of algorithm-guided SDCT is that it requires the herd to be participating in a DHIA testing program, and be accurately detecting clinical mastitis events and recording them into electronic herd records.

1.14 Economic Considerations for Different Dry Cow Therapy Strategies

One final, important consideration for SDCT is the economic impact. This is important because economics are potent motivators for dairy producers (Valeeva et al., 2007). The

economic advantage of SDCT is that it provides an opportunity for the farmer to reduce drug expenditure at dry-off. However, for SDCT to have a positive net-financial impact, these savings must offset the additional expenditure associated with selecting cows for treatment (eg. sampling and culturing milk samples, monthly testing of SCC). Furthermore, if improperly implemented, SDCT has the potential to increase the incidence of clinical and subclinical mastitis in the subsequent lactation, which if present, would have substantial financial impacts (Rollin et al., 2015).

The economic impact of SDCT has been evaluated using dynamic and stochastic budgets (Berry et al., 2004, Huijps and Hogeveen, 2007, Scherpenzeel et al., 2016, Patel et al., 2017, Scherpenzeel et al., 2018). Huijps and Hogeveen (2007) conducted stochastic economic modelling to compare different DCT strategies in Dutch dairy herds. They found that the differences in health outcomes between DCT approaches was the most influential factor impacting the cost-effectiveness of each approach. Following a large field trial in the Netherlands (Scherpenzeel et al., 2014), the same research group conducted two dynamic economic models to further evaluate SDCT for Dutch dairy herds (Scherpenzeel et al., 2016, Scherpenzeel et al., 2018). In both studies, they found that using algorithm-guided SDCT resulted in a slightly higher net economic return than BDCT, despite slight increases in clinical and subclinical mastitis in cows that were not treated with DCT at dry-off. Culture-guided SDCT was not evaluated in any of the aforementioned studies.

Patel et al. (2017) reported a dynamic partial budget for the economic impact of using a quarter-level culture-guided SDCT program in one U.S. dairy herd. They found that the reduced expenditure from reduced drug use (48%) at dry-off ($9.50 per cow) offset the increased costs incurred to implement a rapid culture system on farm ($6.73), thus yielding a net economic impact of $2.77. Therefore, the findings from that study indicate that culture-guided SDCT could be a more economical approach for U.S. dairy farmers than BDCT. However, the latter was a pilot study of a single herd. Furthermore, the price for treating four quarters with DCT (i.e. BDCT) was assumed be $19.00, which likely represents the upper limit of antibiotic-associated costs with BDCT in commercial dairy herds in the U.S. (NAHMS, 2014b). Therefore, more research is needed to evaluate the economic impact of algorithm-guided SDCT and culture-guided SDCT approaches in U.S. herds that accounts for variability in drug costs, antibiotic use reduction with SDCT, as well as evaluating the potential impact of negative health events due to SDCT (McDougall, 2010, Rajala-Schultz et al., 2011, Scherpenzeel et al., 2014).

1.15 Summary

In conclusion, mastitis is an important disease in dairy cows. The majority of antibiotics used on dairy farms are for the purpose of mastitis control, with many used for DCT. Given that antibiotic stewardship is more important than ever, there is a genuine need to improve DCT practices. However, we need a better understanding of the prevalence and etiology of IMI at dry-off, which are the targets of DCT. Furthermore, research should be conducted to identify risk factors for IMI in late lactation cows, in order to inform control strategies.

Recent trials of SDCT in North America indicate that culture- and algorithm-guided SDCT could potentially be used to reduce antibiotic use at dry-off. However the recent, successful studies conducted in the U.S. have been single herd studies. Therefore, a multi-site trial is needed to evaluate the effect of culture- and algorithm-guided SDCT on antibiotic use at dry-off, dry period IMI dynamics and post-calving health and productivity.

1.16 Objectives

Objective 1. Describe the quarter-level prevalence of IMI in late lactation cows in U.S. dairy herds.

Objective 2: Describe associations between bedding type, bedding bacteria count and intramammary infection risk in late lactation cows.

Objective 3. Determine the effect of selective dry cow therapy on common measures of udder health.

Objective 4. Describe the characteristics of screening tests commonly used in selective dry cow therapy programs.

2 CHAPTER TWO: Cross-sectional Study of the Relationship among Bedding Materials, Bedding Bacteria Counts and Intramammary Infection in Late Lactation Dairy Cows

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2.1 Summary

Objectives of this study were to; 1) describe the intramammary infection (**IMI**) prevalence and pathogen profiles in quarters of cows approaching dry-off in U.S. dairy herds, 2) compare IMI prevalence in quarters of cows exposed to different bedding material types, and 3) identify associations between bedding bacteria count (**BBC**) and IMI in cows approaching dry-off. Eighty herds using one of four common bedding materials (manure solids; **MS**, organic non-manure; **ON**, new sand; **NS** and recycled sand: **RS**) were recruited in a multi-site cross-sectional study. Each herd was visited twice for sampling. At each visit, aseptic quarter-milk samples were collected from 20 cows approaching dry-off (>180 days pregnant). Samples of unused and used bedding were also collected. Aerobic culture was used to determine the IMI status of 10,448 quarters and to enumerate counts (log_{10} CFU / cc) of all bacteria, *Staphylococcus spp.,* *Streptococcus spp.* and Strep-like organisms (**SSLO**), Coliforms, *Klebsiella spp.,* non-coliform Gram-negatives, *Bacillus spp.* and *Prototheca spp.* in unused (n = 148) and used (n = 150) bedding. The association between BBC and IMI was determined using multivariable logistic regression with mixed effects.

Quarter-level prevalence of IMI was 21.1%, which was primarily caused by non-aureus *Staphylococcus spp.* (**NAS;** 11.4%) and SSLO (5.6%). Only modest differences in IMI prevalence were observed between the four common bedding material types. Counts of all bacteria in unused bedding was positively associated with odds of IMI caused by any pathogen (**ALL-IMI**; OR = 1.08). A positive association was also observed for counts of SSLO in unused bedding and SSLO-IMI (OR = 1.09). These patterns of association were generally consistent across the four common bedding materials. In contrast, the association between counts of all bacteria in used bedding and ALL-IMI varied by bedding type, with positive associations observed in quarters exposed to MS (OR = 2.29) and ON (OR = 1.51) and a negative association in quarters exposed to NS (OR = 0.47). Findings from this study suggest that quarter-level IMI prevalence in late lactation cows is low in U.S. dairy herds. Furthermore, bedding material type may not be an important risk factor for IMI in late lactation. Higher levels of bacteria in bedding may increase IMI prevalence at dry-off in general, but this relationship is likely to vary according to bedding material type.

2.2 Introduction

Cows acquire intramammary infections (**IMI**) during lactation, some of which can persist through the dry period to impact udder health in subsequent lactations (Green et al., 2002). To cure these IMI, intramammary antimicrobial treatments are administered to cows at the time of dry-off (dry cow therapy; **DCT**). However, there is interest within the dairy industry to reduce antimicrobial use, and DCT is a substantial contributor to antimicrobial use on dairy farms (Pol and Ruegg, 2007, Redding et al., 2019).

Antimicrobial use could be reduced by employing more efficient DCT strategies (such as selective DCT) and by reducing the acquisition of new IMI during lactation, thereby reducing the proportion of cows requiring antimicrobial treatment at dry-off. However, the absence of a nation-wide survey of IMI at dry-off in the U.S. impedes the development and widespread adoption of more efficient DCT programs. Furthermore, additional research is needed to determine risk factors for IMI at dry-off. Molecular epidemiologic studies have identified IMI-causing strains of bacteria in bedding material, suggesting that bedding can act as a reservoir for some pathogens (Verbist et al., 2011, Eraclio et al., 2018), and aerobic culture of bedding to determine bedding bacteria count (**BBC**) has been used to approximate bedding-associated mastitis risk (Hogan et al., 1989). However, few studies have demonstrated a clear association between BBC and udder health, and none have evaluated the association between BBC and IMI in late lactation cows. Hogan et al. (1989) reported a positive association between counts of Gram-negative bacteria and *Klebsiella spp.* in bedding and clinical mastitis incidence in nine U.S. dairy herds. However, no associations were found with other pathogens, including environmental *Streptococcus spp*., which were a common cause of clinical mastitis in that study. Thomas et al. (1983) identified a positive association between BBC and clinical mastitis incidence for *Klebsiella spp*. but not *E. coli*. Similarly, a longitudinal study of herds using manure solids bedding found that when *Klebsiella spp.* counts exceeded 6.0 $\log_{10}$ CFU / g, the proportion of clinical cases caused by *Klebsiella spp.* increased (Carroll and Jasper, 1978). Natzke and LeClair (1976) failed to increase the incidence of new IMI in a small sample of cows, after inoculating their sawdust bedding with *E. coli* for four weeks.

There is indirect evidence to suggest that high levels of bacteria in bedding can negatively affect udder health. Many studies have demonstrated a correlation between BBC and teat end bacteria count (Hogan and Smith, 1997, Hogan et al., 1999, Zdanowicz et al., 2004, Proietto et al., 2013, Rowbotham and Ruegg, 2016b). Extrapolating these associations to udder health is problematic, as no studies to our knowledge have demonstrated a strong association between teat end bacteria counts and mastitis. Consequently, there is currently a lack of rigorous evidence to support the widely held belief that high BBC is a risk factor for IMI and mastitis. Demonstrating an association between BBC and IMI would help to validate BBC as a diagnostic or monitoring tool, and increase the relevance of studies that have investigated methods to reduce BBC (Hogan and Smith, 1997, Hogan et al., 1999, Godden et al., 2008, Bey et al., 2009, Rowbotham and Ruegg, 2016b).

In addition, bedding-related research is needed to address pragmatic questions facing dairy producers. Producers in confinement-managed herds need to know the advantages and disadvantages of different bedding material systems, especially with regard to the commonly used bedding materials: manure solids (**MS**), new sand (**NS**), recycled sand (**RS**) and organic non-manure (**ON**) bedding. It is generally expected that farms using bedding material types with higher BBC, such as MS, may have a higher prevalence of IMI or a different profile of pathogens causing IMI. Furthermore, certain bedding material types may exhibit synergism with BBC in the development of IMI. These hypotheses are based on findings from observational studies where bedding bacterial populations and udder health outcomes varied according to bedding material type (Hogan et al., 1989, Zdanowicz et al., 2004, Bey et al., 2009, Rowbotham and Ruegg, 2015,

2016a, b). For example, a prospective cohort study reported by Rowbotham and Ruegg (2016b) found that NS bedding had the lowest counts of *Streptococcus* and Strep-like organisms (**SSLO**), Gram-negative and coliform bacteria, when compared with RS and MS. In the same study, primiparous cows exposed to NS had significantly lower teat end coliform counts and a tendency for a lower incidence rate of clinical mastitis than cows exposed to other bedding materials (Rowbotham and Ruegg, 2016a). A cross-sectional study of 325 Wisconsin dairy herds by the same research group also found that herds using sand bedding had lower somatic cell counts (**SCC**) and higher milk production than herds using organic bedding materials (Rowbotham and Ruegg, 2015). In summary, more research is needed to understand the prevalence of, and bedding-related risk factors for IMI in cows approaching dry off. Improving our knowledge in this area could lead to improvements in bedding management, reductions in IMI and more efficient approaches to DCT.

The objectives of this study were to; 1) describe the IMI prevalence and pathogen profiles in quarters of cows approaching dry-off in U.S. dairy herds, 2) compare IMI prevalence in quarters of cows exposed to different bedding material types, and 3) identify associations between BBC and IMI in cows approaching dry-off.

2.3 Materials and Methods

The Strengthening the Reporting of Observational Studies in Epidemiology – Veterinary Extension (STROBE-Vet) statement guidelines were followed in the reporting of this study (Sargeant et al., 2016). A cross-sectional study of U.S. dairy herds was conducted between August 2017 and April 2018.

2.3.1 Study Herds

A convenience sample of 80 herds from 10 dairy states were selected. Herd eligibility criteria included; milking herd size greater than 200 cows, have a working relationship with the University of Minnesota or a local Zoetis Quality Milk Specialist (n = 9), and be using one of four bedding materials: MS, NS, ON or RS. In July 2017, a list of eligible herds (n = 152) was created and 80 were selected using a randomized, stratified sampling method, in attempt to enroll an equal number of herds using each bedding type and to maximize the representation of each bedding type within U.S. dairy regions (Northeast, Midwest, Northwest and Southwest).

2.3.2 Farm Visits for Sample and Data Collection

Each farm was visited once during summer (August to September 2017) and once during winter (December 2017 to April 2018). At each visit, aseptic quarter milk samples were collected from 20 late gestation cows (> 180 days pregnant), as well as used and unused bedding from the pens of sampled cows. Farms located in Minnesota (n = 10) were visited by the authors and veterinary students from the University of Minnesota. All remaining farms (n = 70) were visited by Zoetis Quality Milk Specialists. At each visit, study personnel followed a standardized sample collection protocol. Cow eligibility criteria for selection included the following; lactating, managed in the same bedding environment for the past 100 days, greater than 180 days pregnant and not in the hospital pen on the day of visit. After creating a list of all eligible primiparous and multiparous cows, up to 20 cows were selected, with a view to enrolling 30% primiparous and 70% multiparous cows, respectively. Duplicate, aseptic milk samples were collected from each

functional quarter of enrolled cows according to NMC guidelines (NMC, 2017). Briefly, after milking staff performed their usual pre-milking teat disinfection routine, investigators, wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs, discarded three squirts of foremilk and sampled approximately 20-30 ml of milk into sterile 60-ml vials. Samples were immediately chilled on ice.

For sampling of unused bedding material, investigators collected 20 handfuls from various sections of the pile into a disinfected bucket. Bedding in the bucket was then thoroughly mixed and a subsample (approximately 1 L) was transferred into a zip-lock bag, air expressed and then sealed. In dry-lot herds (n = 2), no unused bedding was collected, and used bedding was collected from the pens of enrolled late lactation cows, in the same manner as described above. In freestall herds, one handful of used bedding was collected from the top 5 cm of material in the back third of at least 20 stalls in the late lactation pen. Care was taken to avoid obvious manure pats when sampling. A subsample was collected into a zip-lock bag from the bucket, in the same way described for unused bedding. The bucket was disinfected between the sampling of used and unused bedding and investigators used new gloves before handling bedding material. Bedding and milk samples were immediately chilled and stored at -20°C at the study site before being freighted overnight on ice to the Laboratory for Udder Health (**LUH**) at the Veterinary Diagnostic Lab, University of Minnesota (St. Paul, MN). Samples were stored at the LUH at -20°C and processed within 8 weeks of collection. At each visit, study investigators completed a questionnaire with the farm manager or owner, to obtain

information about bedding management practices, parlor routines and herd demographics.

2.3.3 Microbiological Culture of Milk and Bedding Samples

2.3.3.1 Milk Culture

The IMI status of each quarter was determined using standard microbiologic methods. Milk samples were thawed at room temperature, homogenized by gentle inversion and plated onto Columbia CNA agar with 5% sheep blood (**CNA**) and MacConkey agar (**MAC**). Agar plates were inoculated with one loop-full (approximately 10µl) of sample, using disposable plastic loops and incubated in aerobic conditions and at 37 ± 2°C for 42-48 hours. Only one sample from each quarter was cultured unless this first sample was contaminated. Samples were classified as contaminated if more than two isolates were recovered. Isolates were identified using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Microflex; Bruker Daltonics Inc, Billerica, MA). Peaks produced by each isolate were analyzed by the MALDI-TOF Biotyper reference library. The confidence level for each diagnosis reported by the software was used in the following fashion; >2.0, species level diagnosis recorded; 1.8 – 2, genus level diagnosis recorded; <1.8, MALDI-TOF diagnosis not recorded and traditional identification methods used. Diagnoses obtained using MALDI-TOF were further evaluated by comparing peaks to a database of commonly isolated mastitis pathogens that have been internally validated at the LUH using 16S sequencing. Traditional identification methods included; differential growth on selective media, colony morphology, catalase reaction, Gram-stain and cytology. To improve the specificity of IMI classification (i.e. reduce false positives), non-aureus *Staphylococcus*

spp. (**NAS**) isolates with less than 2 colonies (<200 CFU/ml) and *Bacillus spp*. isolates with less than 5 colonies (<500 CFU/ml) were reclassified as 'no growth' and the quarter considered uninfected (Dohoo et al., 2011b). This adjustment was made because poor specificity is a more potent source of biased measures of association than poor sensitivity (Haine et al., 2018).

2.3.3.2 Bedding Bacteria Count

Bedding samples were thawed at room temperature before a 50cc sub-sample was weighed and transferred to a sterile plastic bag (Whirl-Pak; Nasco, Fork Atkinson, WI, USA), along with 250 ml of sterile water to create a 1:5 dilution. After the bedding-water mixture was allowed to stand at room temperature for 10 minutes, the bag was agitated and 200µL of four dilutions (1:5, 1:50, 1:500 and 1:5000) of the resulting bedding suspension were inoculated onto CNA and MAC agars as a lawn. Cultures were incubated in aerobic conditions at 37± 2°C for 42-48 hours before reading. At reading, bacteria groups were identified using visual inspection by an experienced microbiologist and enumerated from the dilution plate with the optimal number of colonies (25 to 250 per plate) as being; *Bacillus spp*., *Staphylococcus spp.*, SSLO, coliforms, *Klebsiella spp.* non-coliform Gram-negatives or *Prototheca spp*. MALDI-TOF was used to confirm the identity of representative colonies. The counts from each bacteria group were summed to determine total bacteria count. Colony forming units per cc of bedding, per gram of wet bedding and per gram of dry bedding were recorded. Dry matter percentage was determined by drying duplicate 2g sub-samples at 100°C for 24 h.

2.3.4 Statistical Analysis

2.3.4.1 Sample Size Calculation.

Sample size calculations were performed using herd-level measures of IMI prevalence (i.e. percentage of quarters infected per herd), however analysis was eventually conducted using a quarter-level outcome (infected: Y/N). We estimated that 20 herds per bedding material type were necessary to detect a five percentage point difference in prevalence of Gram-negative IMI between any two bedding types (e.g. MS vs. NS). The following assumptions were used in the sample size calculation: alpha = 0.05, power = 80%, prevalences of 0.05 and 0.10 in reference and comparison herds, standard deviation of IMI prevalence = 0.028. The calculated sample size was further inflated to account for multiple comparisons and the exclusion of ineligible herds and quarters.

2.3.4.2 Variable Management

Herd demographic information, bedding management practices and laboratory findings were recorded in spreadsheets (Google Sheets; Mountain View, CA and Microsoft Excel; Redmond, WA) and imported into the R Statistical Programming Environment (R Core Team, 2018) for analysis. Quarters without a determined bacteriological status, mostly due to contamination of milk samples, were excluded from analysis. Normality of continuous variables was assessed by visualizing normal quantile-quantile plots. For analysis, BBC values greater that zero were $\log_{10}$ transformed, while zero counts received a value of $\log_{10}$(0.5 * the detection limit of the test) or 1.1 $\log_{10}$ CFU per cc. Assigning values of 0.5 * the detection limit, although somewhat arbitrary, can reduce bias associated with limitations in diagnostic tests (Lubin et al., 2004). Region was dichotomized into “east” (IN, MI, MN, NY, WI) and “west” (CA, ID, OR, TX, WA),

which is consistent with other surveys of U.S. dairy farms (USDA-NAHMS, 2014). Correlations between explanatory variables were determined using Pearson's correlation coefficient and Kendall's Tau for normally and non-normally distributed continuous variables respectively. Highly correlated variables (> 0.7) were not offered to the same model, with the more suitable variable chosen based on missing values, reliability of measurement or biological plausibility.

2.3.4.3 Association between Bedding Bacteria Count and Intramammary Infection

A directed acyclic graph illustrates the general model building approach used for this objective (Figure 2.1). Multivariable logistic regression models with mixed effects were used to determine associations between BBC and odds of quarter-level IMI using the "glmer" function, with the "bobyqa" optimizer, in the "lme4" package. The three outcome variables of interest included: ALL-IMI, NAS-IMI and SSLO-IMI. ALL-IMI cases were defined as quarters infected with at least one pathogen. Non-aureus *Staphylococcus spp.* NAS-IMI cases were defined as quarters infected with any *Staphylococcus spp.* other than *Staphylococcus aureus*, with all remaining quarters classified as a non-case. SSLO-IMI cases included quarters infected with *Aerococcus, Enterococcus, Lactococcus* and *Streptococcus* species, with all remaining quarters classified as non-cases. We also attempted to develop models to describe associations between BBC and IMI caused by coliforms, but these were abandoned because; 1) the prevalence of coliform IMI was very low and thus associations were unlikely to be of biological significance and 2) multivariable models failed to converge, possibly due to small number of cases.

For each of the three IMI outcomes, the corresponding BBC measure was used as the primary explanatory variable of interest. For the ALL-IMI models, $\log_{10}$ CFU/cc of total bacteria count per cc of bedding was used. For the NAS-IMI and SSLO-IMI models, $\log_{10}$ CFU/cc of *Staphylococcus spp.* and SSLO counts were used, respectively. For comparison, equivalent models that included BBC measures in alternative units (CFU / g and CFU / g DM) were also created. In these models, the BBC measure was included as a continuous variable. As an exploratory analysis, additional models were developed using various categorical BBC variables that were created by collapsing BBC into two, three or four levels based on percentiles. For example, BBC was dichotomized at the 60^{th} percentile, to create a 'high' and 'low' group. The hierarchical structure of the data was addressed in all models, by fitting random intercepts for cow and herd, such that quarters were nested within cows, and cows within herds. Other explanatory variables were offered in multivariable models to account for confounding, and to identify independent predictors of the outcome of interest. Potential cow-level covariates included days pregnant, parity (grouped into 4 categories: 1, 2, 3, ≥4) and breed (Holstein or non-Holstein). The remaining potential covariates were measured at the herd-level, including; season, region, herd average daily milk production (kg/cow/day), bedding material type, frequency of bedding replacement, herd size, paddock access, milking schedule, unit washing practices, use of forestripping, use of udder clipping and use of tail hair management (e.g. docking or trimming switches). Bedding type was expected to be a confounder of the association between BBC and IMI and thus, was forced into all models that included multiple bedding types as a fixed effect. Other potential covariates were initially evaluated using univariable logistic regression with mixed effects, with those

associated (type 2 Wald Chi-Square test at $P < 0.2$) with the outcome of interest being offered to the multivariable model in the first step. The covariates with the highest P-values were removed, one at a time from the model in successive steps, until all remaining fixed effects had type 2 Wald Chi-square tests at $P < 0.05$. Bedding bacteria count was forced into the final model, regardless of P-value. Biologically plausible two-way interactions on the multiplicative scale were investigated and retained in the model if the Wald test for the interaction was $P < 0.05$. Model fit was assessed by comparing regression curves (produced with estimated marginal means) to the raw prevalence of IMI at various values of BBC using a smoother.

2.3.4.4 Association between bedding type, season and intramammary infection

Prevalences of IMI at the quarter- and herd-level were calculated. Models were built using the same methods described earlier to determine associations between the explanatory variables bedding material type and season and the outcome variables ALL-IMI, NAS-IMI and SSLO-IMI. Only crude prevalences are reported in this study and all odds ratio estimates are derived from multivariable models (i.e. they are adjusted for covariates).

2.4 Results

2.4.1 Enrollment

The median number of milking cows was 1,820 (235 to 9,650) and the average daily milk production was 38 (23 to 48) kg per cow. Herds were enrolled from the following states: California (n = 16), Idaho (n = 6), Indiana (n = 4), Michigan (n = 5), Minnesota (n = 10), New York (n = 9), Oregon (n = 1), Texas (n = 2), Washington (n = 6) and Wisconsin (n = 21). Enrolled cows were either Holstein (n = 2678, 85%) or other (n = 480, 15%), which included Holstein-cross, Jersey, Brown-Swiss and their crosses. Housing systems used for lactating cows included freestall (n = 73, 91%), dry lot (n = 2, 3%) and combinations of either freestall and dry lot (n = 4, 5%) or freestall and bedded back (n = 1, 1%). In herds with combination bedding systems, only cows using freestalls were enrolled.

A total of 12,345 quarters from 3,158 cows from 80 herds were initially selected into the study. Of these, 785 quarter samples collected at 10 farm visits, from 8 herds, were excluded from analysis either because of a recent change in bedding material type (< 100 d), or because the herd was using a bedding material type that did not meet the inclusion criteria. One herd changed bedding system between visits, but was retained in the study because each visit met inclusion criteria. A further 9.6% (n = 1,112) of quarters were excluded from analysis because of contaminated milk samples. Consequently, 10,448 quarters from 2,889 cows from 78 herds were available for the final analysis.

2.4.2 Intramammary Infection

The quarter-level prevalence of ALL-IMI was 21.05% (2,199 / 10,448; Table 2.1). 50.4% of cows had at least one quarter with an IMI. The quarter-level prevalence of IMI caused by pathogen groups were NAS (11.4%), *Staphylococcus aureus* (0.4%), SSLO (5.6%),

other Gram-positive bacteria (3.9%), Gram-negative bacteria (0.8%) and other pathogens (0.2%). Note that these prevalences do not sum to the overall prevalence of IMI (21.05%) because some quarters were infected with two pathogens (n = 147). The most common bacterial species was *Staphylococcus chromogenes*, which infected 6.9% of quarters. Other common causes of IMI included *Aerococcus spp.* (2.0%), *Bacillus spp.* (1.9%), *Lactococcus spp.* (1.8%), and *Corynebacterium spp.* (1.2%). *Staphylococcus aureus* (0.4%), *Streptococcus dysgalactiae* (0.3%), *Streptococcus uberis* (0.3%), coliforms (0.1%) and *Prototheca spp.* (<0.1%) were uncommon causes of IMI. Intramammary infection prevalence at the herd-level was similar to that observed overall. The average proportion of infected quarters per herd was 21.5% (Standard deviation; SD = 9.5%) for all IMI, 11.3% (SD = 6.7%) for NAS, 4.8% (SD = 4.8%) for SSLO and 0.1% (SD = 0.4%) for coliforms (Table 2.2). The average proportion of cows with at least one infected quarter per herd was 50.5% (SD = 13.8%) for all IMI, 29.5% (SD = 13.7%) for NAS, 16.8% (SD = 11.7%) for SSLO and 0.5% (SD = 1.3%) for coliforms. The interclass correlation coefficient (a statistic used to describe clustering of hierarchical data) for herd and cow were 0.06 and 0.18 respectively, when fitting a random effects model for ALL-IMI.

2.4.3 Bedding Bacteria Count

One hundred forty-eight unused and 150 used bedding samples from eligible herds were included in analysis. Median bacteria counts ($\log_{10}$ CFU / cc) for each pathogen group for MS, NS, ON and RS are shown in Table 2.3 and in Figures Figure 2.2 and Figure 2.3. All methods for reporting BBC ($\log_{10}$ CFU per cc, $\log_{10}$ CFU per g wet and $\log_{10}$ CFU per g DM) were highly correlated with each other (Kendall's Tau > 0.87). Consequently, only

the results using $\log_{10}$ CFU per cc are reported. The median (interquartile range; **IQR**) total bacteria count in unused bedding was MS (6.28; 5.34 – 6.80), NS (4.31; 4.04 – 4.83), ON (3.17; 1.88 – 4.88) and RS (5.90; 5.22 – 6.39). For used bedding, the median (IQR) total bacteria count was MS (6.95; 6.88 – 7.12), NS (6.91; 6.85 – 7.11), ON (6.66; 6.04 – 6.90) and RS (6.85; 6.80 – 6.94). The proportion of unused bedding samples with no bacterial colonies isolated (reported as 1.1 $\log_{10}$ CFU / cc in Table 2.3) was MS (0%), NS (0%), ON (12.5%) and RS (0%). Bacterial colonies were isolated from all used bedding samples. The proportion of unused bedding samples with no *Staphylococcus spp.* colonies was MS (55.0%), NS (56.8%), ON (53.1%) and RS (59.0%). The proportion of used bedding samples with no *Staphylococcus spp.* colonies was MS (26.2%), NS (73.0%), ON (15.6%) and RS (43.6%). The proportion of unused bedding samples with no SSLO colonies was MS (47.5%), NS (51.4%), ON (65.6%) and RS (33.3%). The proportion of used bedding samples with no SSLO colonies was MS (14.3%), NS (13.5%), ON (9.4%) and RS (7.7%). No *Prototheca spp.* were isolated in any bedding samples. The proportion of used bedding samples with counts of SSLO at the upper detection limit (6.80 $\log_{10}$ CFU / cc) was MS (28.6%), NS (29.7%), ON (18.8%) and RS (7.7%).

2.4.4 Association between Bedding Bacteria Counts and Intramammary Infection

Odds ratio estimates of the associations between counts of all bacteria, *Staphylococcus spp.* ad SSLO in bedding and odds of ALL-IMI, NAS-IMI and SSLO-IMI respectively, are shown in Figures Figure 2.4, Figure 2.5 and Figure 2.6. Similar associations were identified when using alternative BBC units (CFU per g wet or CFU per g dry matter) or when categorical BBC variables were used. Consequently, only estimates from models

using BBC as a continuous predictor, measured on the $\log_{10}$ CFU per cc scale are reported.

2.4.4.1 Unused bedding

In unused bedding, total bacteria count was associated with odds of ALL-IMI (OR = 1.08; 95% CI: 1.00 – 1.17, Figure 2.4), as was SSLO count and SSLO-IMI (OR = 1.09; 95% CI: 1.00 – 1.19, Figure 2.6), but not *Staphylococcus spp.* count and NAS-IMI (OR = 0.98; 95% CI: 0.91 – 1.05, Figure 2.5). There was no statistical evidence of effect modification by bedding type in unused bedding (Wald test for BBC * bedding type interaction term was $p > 0.05$ in models for ALL-IMI, NAS-IMI and SSLO-IMI). Nevertheless, stratified models were created. Odds ratio estimates were generally consistent across the four common bedding types for the association between total bacteria count and ALL-IMI and *Staphylococcus spp.* count and odds of NAS-IMI. The associations between SSLO count and SSLO-IMI were more variable between bedding types, with positive odds ratio estimates in MS (OR = 1.06; 95% CI: 0.78 – 1.45), ON (OR = 1.55; 95% CI: 1.18 – 2.03) and RS (OR = 1.13; 95% CI: 1.02 – 1.25) and a negative association for NS (OR = 0.82; 95% CI: 0.68 – 0.99).

2.4.4.2 Used bedding

The association between BBC in used bedding and odds of IMI varied considerably by bedding type (Wald test for interaction terms for BBC * bedding type was $p < 0.05$ in models for ALL-IMI, NAS-IMI and SSLO-IMI). Main effects models (i.e. models omitting the interaction with bedding type) indicated an overall positive association between total bacteria count and ALL-IMI (OR = 1.41; 95% CI: 1.11 – 1.81, Figure 2.4),

and possibly between *Staphylococcus spp*. and NAS-IMI (OR = 1.05; 95% CI: 0.99 – 1.10, Figure 2.5) and between SSLO and SSLO-IMI (OR = 1.07; 95% CI: 0.99 – 1.16, Figure 2.6). However, odds ratio estimates for the association between total bacteria counts in used bedding and odds of ALL-IMI, after stratifying by bedding type, indicated a positive association in quarters exposed to MS (OR = 2.29; 95% CI: 1.15 – 4.54) and ON (OR = 1.51; 95% CI: 1.09 – 2.09), and a negative association in quarters exposed to NS (OR = 0.47; 95% CI: 0.26 – 0.87). In quarters exposed to RS, total bacteria count in used bedding and odds of ALL-IMI showed little evidence of association (OR = 0.83, 95% CI: 0.54 – 1.27). A similar pattern of effect modification was evident for the association with *Staphylococcus spp*. count in used bedding and odds of NAS-IMI, with positive associations in quarters exposed to MS (OR = 1.10; 95% CI: 1.02 – 1.19) and ON (OR = 1.18; 95% CI: 1.05 – 1.32), and negative association in quarters exposed to NS (OR = 0.83; 95% CI: 0.72 – 0.95). There was little evidence of an association between *Staphylococcus spp.* count in used bedding and odds of NAS-IMI in quarters exposed to RS (OR = 0.98; 95% CI: 0.88 – 1.09). Estimates for the association between SSLO count in used bedding and odds of SSLO-IMI were less precise than other models, with little evidence for associations among quarters exposed to MS (OR = 0.95; 95% CI: 0.69 – 1.30), NS (OR = 0.95; 95% CI: 0.85 – 1.07) and RS (OR = 1.10; 95% CI: 0.95 – 1.26). The association between SSLO count in used bedding and odds of SSLO-IMI in quarters exposed to ON bedding was positive (OR = 1.61, 95% CI: 1.20 – 2.16).

2.4.5 Association between Bedding Type, Season of Sampling and Intramammary Infection

Only modest differences in IMI prevalence were found when comparing quarters exposed to MS (19.3%), NS (23.9%), ON (22.7%) and RS bedding (19.0%; Table 2.1). Odds of SSLO-IMI was lower in MS-exposed quarters (2.0%) when compared with quarters exposed to other bedding material types: RS (7.3%, OR = 3.53, 95% CI: 2.23 – 5.58), NS (7.1%; OR = 3.53, 95% CI: 2.28 – 5.47) and ON (6.7%; OR = 3.29, 95% CI: 2.10 – 5.16; Table 2.4). *Lactococcus spp.* IMI were higher in quarters exposed to NS (3.3%) and RS (3.4%) than those exposed to MS (0.2%) and ON (0.3%). Exploratory analysis with multivariable logistic regression with mixed effects found that quarters exposed to inorganic bedding (NS or RS) had much higher odds of *Lactococcus spp.* IMI than quarters exposed to organic (MS or ON) bedding (OR = 11.02; 95% CI: 2.70 – 30.42). Prevalence of NAS-IMI was somewhat similar between bedding types except for a lower prevalence in quarters exposed to RS than MS (9.2% vs 12.7%; OR = 0.56, 95% CI: 0.36 – 0.87). Quarters sampled during winter 2017-18 had a lower prevalence of IMI (18.9%, OR = 0.75; 95% CI: 0.66 – 0.84) than quarters sampled during summer 2017 (23.1%). This was due to a lower prevalence of NAS-IMI in the winter period (9.6% vs 13.10%, OR = 0.64; 95% CI: 0.54 – 0.76).

2.5 Discussion

2.5.1 Prevalence of Intramammary Infection in Late Lactation Cows

To our knowledge, this is the largest survey of IMI in cows approaching dry-off in the U.S. The quarter-level prevalence of ALL-IMI was 21.1%. In other recent surveys of cows at dry-off in U.S. herds, the prevalence of IMI was 12.8% (Pantoja et al., 2009),

19.2% (Arruda et al., 2013) and 34.7% (Johnson et al., 2016). The prevalence found in our study is likely to be a more accurate prevalence estimate than the aforementioned studies, which only enrolled 11 herds between them. The low prevalence found in this study indicates that antibiotic use at dry-off could be reduced in North American dairy herds by implementing selective DCT (Cameron et al., 2014, Vasquez et al., 2018). The finding that 50.5% of cows in our study had at least one infected quarter (compared to the 21.1% quarter-level prevalence) indicates that implementing selective DCT at the quarter-level (Patel et al., 2017) could reduce antibiotic treatments more than programs implemented at the cow-level (Vasquez et al., 2018). To our knowledge, antibiotic reductions from quarter and cow-level selective DCT approaches have not been compared within a single study. More than half of ALL-IMI were caused by NAS (11.4%), which are likely to have a high spontaneous dry period cure risk (Vasquez et al., 2018) and often have minimal effects on clinical mastitis risk (Green et al., 2002) or milk production in the subsequent lactation (Vanderhaeghen et al., 2014). More research is needed to understand the effect of NAS-IMI at dry-off on udder health in the subsequent lactation, so that selective DCT programs can be optimized to either treat or withhold treatment from NAS infected quarters.

The prevalence of SSLO in this study was 5.6%, which consisted of *Aerococcus spp.* (2.0%), *Lactococcus spp.* (1.8%), *Streptococcus spp.* (1.1%) and *Enterococcus spp.* (0.7%). Distinguishing between genera and species within the SSLO category has recently improved with the inclusion of MALDI-TOF in diagnostic laboratories. Research is needed to describe the contrasting epidemiologic characteristics of these pathogens because most epidemiologic studies of SSLO IMI were conducted using

traditional laboratory methods. Recent research suggests that they are not a homogenous group. For example, reports have suggested that *Aerococcus spp.* is a teat apex commensal (Braem et al., 2012) and that IMI caused by this pathogen do not have an effect on udder health (Wyder et al., 2011). In contrast, case reports have documented herd outbreaks of clinical mastitis caused by *Lactococcus spp.* (Werner et al., 2014, Rodrigues et al., 2016).

In our study, IMI in late lactation cows were mostly caused by Gram-positive bacteria, which accounted for 96.0% of infected quarters. Interestingly, only one quarter was infected with *Prototheca spp.* and no colonies were isolated from bedding, indicating that the herd-level prevalence of *Prototheca spp*. may be low in late lactation cows in U.S. dairy herds. However, specialized medias were not used in the culture of milk or bedding, which may have reduced detection sensitivity. In our study, all cultures were performed by an experienced microbiologist using medias that can successfully isolate *Prototheca spp.* (Lass-Flörl and Mayr, 2007). Previous studies have isolated *Prototheca spp*. from bedding material, but the importance of bedding material in the pathogenesis of *Prototheca* mastitis is still unclear (Ricchi et al., 2010).

Less than 1% of quarters were infected with Gram-negative bacteria, which accounted for only 3.3% of infected quarters (Table 2.1). Furthermore, only 0.14% quarters were infected with coliform bacteria overall, and the average herd prevalence of coliform infected quarters and cows was also very low, which was consistent across all herds in this study (Table 2.2). Longitudinal studies of IMI in dairy cattle have shown that the prevalence of IMI caused by coliforms decreases as cows approach dry-off (Smith et al., 1985, Bradley et al., 2015). Other surveys of IMI at dry-off have also reported a very low

prevalence of coliform infections, including 0.9 (Hogan et al., 1989), 1.27 (Bradley et al., 2015), 2.2 (Arruda et al., 2013) and 2.7% (Johnson et al., 2016).

The low prevalence of coliform IMI in our study, and others, suggests that dry cow therapy in U.S. dairy herds should primarily target Gram-positive bacteria, and may explain why IMI cure risk during the dry period appears to be similar when using narrow or broad-spectrum antimicrobials (Arruda et al., 2013, Johnson et al., 2016). However, some herds may benefit from the use of broad-spectrum antimicrobials at dry-off. One small trial conducted by Pinedo et al. (2012) in two Florida herds found that treatment with a narrow spectrum antibiotic (1,000,000 IU of procaine penicillin G and 1 g of dihydrostreptomycin) resulted in higher incidences of clinical and subclinical mastitis in the first 60 days after calving, as compared to treatment with a broad spectrum product (500 mg ceftiofur hydrochloride). However, those results differ from a larger randomized trial conducted in six herds in four states, which showed no difference in subclinical or clinical mastitis risk in the first 100 days of lactation in cows treated with the same two antibiotics (Arruda et al., 2013b).

2.5.2 Bedding Type Choice

Only modest differences in quarter-level prevalence of ALL-IMI were observed in late-lactation cows using four common bedding materials evaluated: MS (19.3%), ON (22.7%), NS (23.9%) and RS (19.0%). This finding is consistent with some studies that have failed to show bedding-associated differences in clinical mastitis incidence (Hogan et al., 1989) and SCC (Eckelkamp et al., 2016). However, other studies have identified associations between bedding type and bulk milk SCC (Ostrum et al., 2008, Rowbotham and Ruegg, 2015) and bacteria counts on teat skin (Rowbotham and Ruegg, 2016b).

Rowbotham and Ruegg (2016a) found that the incidence rate of clinical mastitis (cases per 1000 quarter days at risk) was numerically lower in primiparous cows using NS (0.13) than in those using RS (0.34) and MS (incidence rate ratio = 0.31; Type III P-value from cox proportional hazards model = 0.06). In some cases, MS has been associated with worse udder health (Ostrum et al., 2008). The perceived negative effects of MS bedding on udder health has been mostly attributed to increased concentrations of coliform bacteria (Carroll and Jasper, 1978). As previously mentioned, only late lactation cows were enrolled in our study, which may explain the very low prevalence of IMI caused by coliforms (0.14%). This apparent resistance in late lactation cows to coliform IMI may explain why MS-exposed quarters had similar IMI prevalences to other bedding materials. Studies of early lactation cows may have identified meaningful differences in coliform IMI between bedding types. Interestingly, quarters exposed to MS had a much lower prevalence (2%) of SSLO, than quarters exposed to NS (7.1%), ON (6.7%) and RS (7.3%). This finding cannot be easily attributed to the SSLO count in MS bedding, as it was numerically greater than most other bedding materials in both used and unused samples. The prevalence of *Lactococcus spp*. IMI was greater in quarters exposed to inorganic bedding (NS: 3.3%, RS: 3.4%) than those exposed to organic bedding (MS: 0.2%, ON: 0.3%; OR = 11.02; 95% CI: 2.70 – 30.42). This finding is consistent with recent case reports of *Lactococcus spp.* outbreaks occurring in herds using inorganic bedding (Werner et al., 2014, Rodrigues et al., 2016, Eraclio et al., 2018).

2.5.3 Bedding Bacteria Count was Associated with Intramammary Infection in Late Lactation Cows

The findings from this study are generally consistent with the widely held belief that higher levels of bacteria in bedding material increase the risk of IMI. Three observational studies have shown positive associations between BBC and clinical mastitis incidence (Carroll and Jasper, 1978, Thomas et al., 1983, Hogan et al., 1989). In each study, clinical mastitis incidence was only associated with counts of coliform or *Klebsiella spp.* in bedding. In this study, we were not able to identify associations between levels of coliform bacteria in bedding and coliform IMI, possibly due to the very low prevalence of IMI caused by Gram-negative bacteria (0.84%) and coliforms (0.14%). A reason for this discrepancy between ours and previous studies may be that we were studying prevalence IMI in late lactation cows, while previous studies reported on incidences of clinical mastitis events at any time during the lactation. Given that coliform IMI tend to be short-lived, cohort study designs would be more suitable than cross-sectional to investigate the potential impact of bedding-associated coliform bacteria on clinical mastitis incidence.

Our study is the first to demonstrate an association between counts of all bacteria, *Staphylococcus spp.* and SSLO in bedding and odds of subclinical IMI caused by these specific pathogen groups. However, in a cross-sectional observational study such as this, such associations are not definitive proof of a causal relationship. For example, one could argue the causal relationship could be in the opposite direction, with high IMI prevalence increasing BBC. A study by Piessens et al. (2011) found that *Staphylococcus chromogenes* (the most common cause of IMI in our study) rarely established a reservoir in bedding material, and was more often found in body sites of the cow, such as skin and

the mammary gland. It is therefore possible that a higher prevalence of cows harboring this pathogen as an IMI could increase bacterial contamination of bedding material. This hypothesis is consistent with our findings, as there was only a positive association between *Staphylococcus spp.* count and NAS-IMI in used bedding samples and not in unused samples. It is possible that the association is bidirectional as some species of NAS may have an environmental reservoir, while other species of NAS may have their reservoir in body sites of the cow (Piessens et al., 2011). The positive association between SSLO counts and odds of SSLO-IMI is consistent with conventional understanding of these pathogens. Like NAS, molecular methods have implicated bedding material and other environmental sources as possible reservoirs for these pathogens (Eraclio et al., 2018). However, other studies have shown that some SSLO (especially *Streptococcus uberis*) can exhibit host adaptation and thus establish new IMI from intramammary reservoirs (Davies et al., 2016).

2.5.4 Bedding Bacteria Count in Unused Bedding and Intramammary Infection were Positively Associated in Most Bedding Material Types

Total bacteria count in unused bedding was positively associated with odds of ALL-IMI (OR = 1.08, 95% CI: 1.00 – 1.17), as were SSLO counts and odds of SSLO-IMI (OR = 1.09, 95% CI: 1.00 – 1.19). These associations were generally positive across all bedding types, except for a negative association between SSLO counts and SSLO-IMI in NS exposed quarters (OR = 0.82; 95% CI: 0.68 – 0.99). The absence of a positive association in NS is not surprising, given that the SSLO in unused NS bedding would not have originated from cows, which is in contrast to RS (OR = 1.13; 95% CI: 1.02 – 1.25), which would contain many SSLO from cow sources like fecal material.

2.5.5 The Association between Bacteria Count in Used Bedding and Intramammary Infection Varied by Bedding Material Type

In contrast to unused bedding, we found that that the relationship between total bacteria count and odds of ALL-IMI in used bedding samples was inconsistent across bedding material types. Models evaluating the relationship between counts of all bacteria in bedding and ALL-IMI found positive associations in quarters exposed to MS and ON, while no association or negative associations existed for quarters exposed to NS and RS (Figure 2.4). This finding is consistent with a study by Zdanowicz et al. (2004), which found that the correlation between BBC and teat skin bacteria count was higher in teats exposed to sawdust bedding, than in sand bedding. However, that study did not directly investigate risk with IMI. There are a number of potential biological explanations for this effect modification. Firstly, the physical characteristics of bedding materials, such as dry matter, could influence the efficiency of bacterial transmission onto teat skin surfaces. Secondly, this could be a result of bacterial profiles within each pathogen group differing by bedding material type. For example, counts of SSLO were generally higher in MS than in ON bedding. However, the prevalence of SSLO-IMI was higher in quarters exposed to ON (OR = 3.29) than those exposed to MS. Furthermore, the relationship between bedding SSLO count and odds of SSLO-IMI was positive in ON (OR = 1.61), but not in MS (OR = 0.95). Hypothetically, this could be due to a higher proportion of pathogenic SSLO species (such as *Streptococcus dysgalactiae and Enterococcus spp.*) in ON than in MS, thus causing a 1 $\log_{10}$ unit increase in SSLO in ON to be more detrimental to udder health than in MS. There is evidence to suggest that virulence varies significantly within an individual species of bacteria (Zadoks et al., 2011) and so it is

likely that enumerating bacteria at the genus-level will, in some circumstances, lack the necessary precision to predict infection risk, due to the heterogeneity of species within those groups. The development of new laboratory methods to better distinguish between pathogenic and non-pathogenic species would likely improve the utility of bedding culture in research studies, and in mastitis control programs.

In quarters exposed to NS bedding, we found that higher counts of all bacteria and of *Staphylococcus spp*. were associated with lower odds of ALL-IMI and NAS-IMI respectively. Given that this negative association is at odds with basic biological concepts, we are suspicious that this finding may be spurious or biased. For example, selection bias could occur if culling risk was higher in herds with high BBC. However, very little is known about the bacteria communities in bedding, and so our finding could potentially be valid. For example, it is plausible that when bacteria counts in NS are high, they include a high CFU of non-pathogenic bacterial species that exhibit an antagonistic effect on pathogenic bacteria (Westphal et al., 2011). To our knowledge, the manipulation of bacterial communities in bedding material through inoculation has not been studied as a potential mastitis control strategy, but has been trialed in other farming systems (Corrêa et al., 2012). Regardless of the cause, the various associations between BBC and IMI among bedding materials suggests that there is unlikely to be a universal cut-point for bacterial counts that can be applied across all bedding systems.

2.5.6 Utility of Bedding Bacteria Count Measurements as a Bedding Hygiene Monitoring Tool

Bedding culture is an inexpensive test offered by several veterinary diagnostic laboratories in North America. However, bedding culture methods differ among

laboratories and there is no consensus on which methods or reporting units should be used. Bacteria count as CFU has been reported per ml/cc (Bey et al., 2009), per gram (Zdanowicz et al., 2004) and per gram of DM (Hogan et al., 1989). In this study, we found that these measures were highly correlated and consequently delivered equivalent associations with IMI measures. Therefore, each of these measures is likely to be equally useful in research studies or for use in commercial dairy farms. However, variation in testing methods and units does prevent meaningful comparisons of BBC among studies or laboratories that use different bedding culture protocols. We recommend that farmers testing bedding hygiene use a consistent approach (i.e. time of collection, collection technique, laboratory) and monitor for increases in BBC.

2.5.7 Other Determinants of Intramammary Infection

Quarters sampled during the winter period had a lower prevalence of IMI than in summer (OR = 0.75; 95% CI: 0.66 – 0.84). This was due to a lower prevalence of IMI caused by NAS (OR = 0.64; 95% CI: 0.54 – 0.76). Other studies have identified summer as a risk factor for increased SCC in individual cows and bulk milk (Green et al., 2006, Riekerink et al., 2007).

2.5.8 Strengths and Limitations of this Study

One considerable strength of this study is the large sample of herds and cows, which increases the generalizability of the findings and also increases statistical power, thus providing more precise effect estimates (i.e. relatively narrow 95% confidence intervals). However, herds were selected by convenience into this study, which should be considered when generalizing results. Selection criteria included an existing relationship

with the University of Minnesota or Zoetis, which may have biased the selection in favor of larger, well-managed herds. Nevertheless, this is the largest cross-sectional study of BBC and IMI conducted to date, and care was taken to randomly select herds from an eligible pool that included a broad cross-section of herds from all major U.S. dairy regions, using a variety of housing and bedding management systems. Therefore, we believe this study has significant relevance to U.S. dairy herds. Another consideration is that IMI prevalence in late lactation was used as a proxy for IMI prevalence at dry-off. Therefore, we believe that our findings should only be used to complement field trials that investigate the suitability of selective DCT in North American dairy herds. Furthermore, the relationship between bedding type, BBC and IMI in late lactation cows may not be generalizable to other subpopulations of cows (e.g. early or peak lactation). This deserves further study. The reliability of herd-level estimates of prevalence (outlined in Table 2.2) are likely to vary among herds because the sample size for each herd was not adjusted to account for the number of cows. This is unlikely to be a source of error for quarter-level analysis, which accounts for almost all analysis reported.

As already mentioned, cross-sectional studies do not allow for causal inference. Furthermore, establishing exposure and outcome status from samples collected on the same day, can introduce bias. We have assumed that the bacteria count measurements from a single bedding sample collected on the day of visit (the same day that milk samples were collected) was representative of the bacteria counts in the bedding that the quarters were actually exposed to during the period leading up to milk sampling. In our study, the used bedding age (time interval from adding unused bedding to sampling) was not standardized across farms and BBC has been shown to rapidly increase after it is

added to stalls, especially during the first 24hrs (Godden et al., 2008). However, we found that used bedding age was not strongly associated with BBC in our study (+0.02 $\log_{10}$ CFU per day; 95% CI: -0.01 - 0.04) and when included as a covariate to models predicting IMI, the effect estimates and associated confidence intervals did not change appreciably for BBC, suggesting that this unadjusted variability was not a significant source of bias. The median age of used bedding in our study was 2 days (IQR = 1 – 4). Freezing bedding samples could have also introduced measurement error. A recent experiment showed that freezing sand and MS bedding, for 1, 2 or 3 weeks reduced counts of Gram-negative and coliform bacteria, but not other species (Homerosky and Hogan, 2015). It is therefore possible that the counts of Gram-negative and coliform bacteria shown in Table 2.3 may be underestimated. However, given that no associations were made between counts of these species and IMI, it is unlikely that this measurement error has led to significant bias in our study.

Another potential source of measurement error, is the upper detection limit of BBC. As reported, the proportion of used bedding samples with counts of SSLO at the upper detection limit (6.80 $\log_{10}$ CFU / cc) was MS (28.6%), NS (29.7%), ON (18.8%) and RS (7.7%). Therefore, the extremes of SSLO counts (our explanatory variable) were not fully represented in our regression models, which could have biased the association between BBC and IMI toward the null.

2.5.9 Future Research

Cross-sectional studies similar to this that enroll cows from earlier stages of lactation would allow for additional insights into the relationship between bedding hygiene and the development of mastitis during lactation. A small number of observational studies

(including this one) have demonstrated an association between BBC and IMI. However, no studies have shown that reducing BBC improves udder health. Therefore, research is needed to identify best management practices and the cost-effectiveness of interventions designed to reduce BBC. We plan to use the dataset from this study to investigate the relationship between bedding management practices, bedding characteristics (eg. moisture and organic matter concentrations) and bedding bacteria counts.

2.5.10 Conclusion

In a cross-sectional study of late lactation cows from 78 U.S. dairy herds, the prevalence of ALL-IMI was 21.05% of quarters and 50.4% of cows, respectively. Most IMI were caused by NAS and SSLO, with very few quarters infected with coliform bacteria. We found that BBC was positively associated with odds of IMI in general. The association between BBC in unused bedding and IMI was mostly consistent across all four common bedding material types. In contrast, the association between counts of bacteria in used bedding and IMI varied by bedding material type. Only modest differences in IMI prevalence were observed between the four common bedding material types (MS, NS, ON, RS). These results indicate that none of the bedding materials evaluated in this study were superior to another relative to the odds of IMI in late lactation. Further research is necessary to investigate the effects and potential benefits of reducing BBC on udder health.

2.6 Declaration of competing interest

M. Boyle is an employee of the Zoetis. He was involved in the study design and conceptualization with review of the manuscript. He was not involved with any analyses of data. All others have no competing interests to declare.

2.7 Authors roles

SM. Rowe was involved in fieldwork, laboratory work, data management, analysis and manuscript preparation. SM. Godden was involved in supervision, study conceptualization, fieldwork and manuscript editing. E. Royster was involved in study conceptualization and manuscript editing. BA. Crooker was involved laboratory work and manuscript editing. J. Timmerman was involved in laboratory work and manuscript editing. M. Boyle was involved in study conceptualization, fieldwork coordination and manuscript editing.

2.8 Funding

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2.9 Acknowledgements

We thank the Zoetis Quality Milk Specialist and Dairy Technical Services teams (Julio Alcantar, Michele Barrett DVM, Karthryn Browning MS, Ruben Gonzalez, Samuel Herrera, Bernard Kwaku, Shawn Hull, Doris Ledwith DVM, John Lee DVM, Francisco Rivas DVM MS, and Bill Sullivan), who conducted the fieldwork in herds located

outside of Minnesota. We also thank DVM students from the University of Minnesota, Samuel Basquin, Edouard Cotten, Wanda Weber and Aaron Rendahl.

Table 2.1: Quarter-level prevalence of intramammary infection from 2889 late gestation cows, stratified by bedding material exposure

	Manure solids		New Sand		Organic non-manure		Recycled Sand	
	n	%	n	%	n	%	n	%
Total quarters sampled	3286		2842		2450		2982	
Contaminated	221	6.73%	288	10.13%	252	10.29%	351	11.75%
Total quarters at risk (excluding contaminated)	3065	100.00%	2554	100.00%	2198	100.00%	2631	100.00 %
No Growth	2475	80.75%	1943	76.08%	1699	77.30%	2132	81.03%
Infected quarters	590	19.25%	611	23.92%	499	22.70%	499	18.97%
Single pathogen	564	18.40%	570	22.32%	455	20.70%	463	17.60%
Mixed infection	26	0.85%	41	1.61%	44	2.00%	36	1.37%
Gram-Positive[1]	585	19.09%	583	22.83%	463	21.06%	479	18.21%
Staphylococcus aureus	17	0.55%	10	0.39%	9	0.41%	8	0.30%
Non-aureus Staphylococcus spp.	388	12.66%	320	12.53%	238	10.83%	241	9.16%
Staphylococcus chromogenes	297	9.69%	186	7.28%	109	4.96%	133	5.06%
Staphylococcus epidermidis	4	0.13%	13	0.51%	24	1.09%	7	0.27%
Staphylococcus haemolyticus	9	0.29%	16	0.63%	12	0.55%	9	0.34%
Staphylococcus hominis	0	0.00%	0	0.00%	1	0.05%	4	0.15%
Staphylococcus sciuri	20	0.65%	12	0.47%	5	0.23%	2	0.08%
Staphylococcus simulans	8	0.26%	19	0.74%	3	0.14%	33	1.25%
Staphylococcus sp.	48	1.57%	45	1.76%	74	3.37%	56	2.13%
Staphylococcus xylosus	9	0.29%	32	1.25%	15	0.68%	1	0.04%
SSLO	61	1.99%	181	7.09%	147	6.69%	191	7.26%
Aerococcus sp.	3	0.10%	13	0.51%	5	0.23%	13	0.49%
Aerococcus viridans	11	0.36%	46	1.80%	75	3.41%	45	1.71%
Enterococcus faecalis	0	0.00%	2	0.08%	0	0.00%	0	0.00%
Enterococcus hirae	2	0.07%	0	0.00%	0	0.00%	4	0.15%
Enterococcus saccarolyticus	4	0.13%	2	0.08%	6	0.27%	10	0.38%
Enterococcus sp.	5	0.16%	8	0.31%	22	1.00%	7	0.27%
Lactococcus garvieae	2	0.07%	53	2.08%	2	0.09%	70	2.66%
Lactococcus lactis	5	0.16%	24	0.94%	5	0.23%	13	0.49%
Lactococcus sp.	0	0.00%	7	0.27%	0	0.00%	6	0.23%
Streptococcus dysgalactiae	8	0.26%	3	0.12%	18	0.82%	4	0.15%
Streptococcus sp.	6	0.20%	19	0.74%	13	0.59%	14	0.53%
Streptococcus uberis	17	0.55%	5	0.20%	5	0.23%	5	0.19%
Other Gram-positive bacteria	134	4.37%	106	4.36%	96	4.37%	66	2.51%
Arthrobacter gandavensis	0	0.00%	1	0.04%	0	0.00%	0	0.00%
Arthrobacter species	0	0.00%	2	0.08%	0	0.00%	0	0.00%
Bacillus sp.	79	2.58%	46	1.80%	44	2.00%	28	1.06%
Corynebacterium sp.	22	0.72%	41	1.61%	37	1.68%	21	0.80%
Listeria monocytogenes	1	0.03%	0	0.00%	0	0.00%	0	0.00%
Micrococcus sp.	5	0.16%	2	0.08%	0	0.00%	1	0.04%
Trueperella pyogenes	2	0.07%	0	0.00%	1	0.05%	1	0.04%
Gram-positive Cocci	21	0.69%	6	0.23%	8	0.36%	7	0.27%
Gram-positive Rod	5	0.16%	8	0.31%	6	0.27%	8	0.30%
Gram-negative bacteria	5	0.16%	29	1.14%	33	1.50%	21	0.80%
Coliforms	1	0.03%	7	0.27%	3	0.14%	4	0.15%
Citrobacter sp.	1	0.03%	1	0.04%	0	0.00%	1	0.04%
Escherichia coli	0	0.00%	1	0.04%	2	0.09%	0	0.00%
Escherichia sp.	0	0.00%	1	0.04%	0	0.00%	0	0.00%

Klebsiella pneumoniae	0	0.00%	1	0.04%	0	0.00%	0	0.00%
Serratia marcescens	0	0.00%	1	0.04%	1	0.05%	0	0.00%
Serratia sp.	0	0.00%	2	0.08%	0	0.00%	3	0.11%
Other Gram-negative bacteria	4	0.13%	22	0.86%	30	1.36%	17	0.65%
Gram-negative Organism	3	0.10%	8	0.86%	21	0.96%	10	0.38%
Pantoea sp.	0	0.00%	3	0.12%	1	0.05%	2	0.08%
Pseudomonas aeruginosa	0	0.00%	0	0.00%	0	0.00%	1	0.04%
Pseudomonas sp.	0	0.00%	11	0.43%	7	0.32%	4	0.15%
Raoultella sp.	0	0.00%	0	0.00%	1	0.05%	0	0.00%
Stenotrophomonas maltophilia	1	0.03%	0	0.00%	0	0.00%	0	0.00%
Other organisms	1	0.03%	2	0.08%	11	0.50%	4	0.15%
Yeast	1	0.03%	2	0.08%	11	0.50%	3	0.11%
Prototheca sp.	0	0.00%	0	0.00%	0	0.00%	1	0.04%

[1]Reported frequencies of bacteria groups (eg. Gram-positive) are the not the same as the sum of bacteria species within the group due to mixed infections in 147 quarters.

Table 2.2: Herd-level prevalence of intramammary infection in 78 dairy herds in the U.S.

	Infected quarters per herd (%) (n = 78 herds)				Infected cows[1] per herd (%) (n = 78 herds)			
IMI measure	Mean	SD	Min	Max	Mean	SD	Min	Max
All pathogens	21.5	9.4	6.4	52.8	50.5	13.8	20.0	83.3
Non-aureus *Staphylococcus spp.*	11.3	6.7	0.0	37.0	29.5	13.7	0.0	67.6
Streptococcus spp. and Strep-like organisms	4.8	4.8	0.0	26.4	16.8	11.5	0.0	44.4
Coliforms	0.1	0.4	0.0	1.8	0.5	1.3	0.0	5.4

[1]Cows with at least one infected quarter

Note that 40 cows (160 quarters) were sampled from each herd, irrespective of herd size. Therefore, herd-level inference may be limited by this sampling strategy.

Table 2.3: Median (interquartile range) bacteria counts (log CFU/cc) for unused (n = 148) and used (n = 150) bedding samples taken from 78 herds[1].

	Manure solids		New Sand		Organic non-manure		Recycled Sand	
Unused bedding								
Total bacteria count	6.28	(5.34 - 6.80)	4.31	(4.04 - 4.83)	3.17	(1.88 - 4.88)	5.90	(5.22 - 6.39)
Staphylococcus spp.	1.10	(1.10 - 4.28)	1.10	(1.10 - 2.35)	1.10	(1.10 - 2.10)	1.10	(1.10 - 3.49)
SSLO[2]	3.33	(1.10 - 4.91)	1.10	(1.10 - 2.40)	1.10	(1.10 - 2.21)	3.40	(1.10 - 4.88)
Coliforms	1.25	(1.10 - 3.40)	1.10	(1.10 - 1.10)	1.10	(1.10 - 2.92)	1.10	(1.10 - 1.94)
Klebsiella spp.	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)
Non-coliform Gram-negative	4.87	(3.38 - 5.77)	2.00	(1.40 - 2.93)	1.90	(1.10 - 3.82)	4.40	(3.35 - 4.66)
Bacillus spp.	6.11	(5.33 - 6.80)	4.30	(3.83 - 4.81)	2.80	(1.32 - 4.65)	5.81	(5.19 - 6.24)
Used bedding								
Total bacteria count	6.95	(6.88 - 7.12)	6.91	(6.85 - 7.11)	6.66	(6.04 - 6.90)	6.85	(6.80 - 6.94)
Staphylococcus spp.	4.49	(1.17 - 5.47)	1.10	(1.10 - 2.54)	5.01	(2.86 - 5.61)	3.40	(1.10 - 4.88)
SSLO[2]	6.01	(5.38 - 6.80)	5.85	(5.40 - 6.80)	5.83	(5.40 - 6.30)	5.63	(5.18 - 6.01)
Coliforms	4.79	(3.64 - 5.35)	3.65	(3.32 - 4.35)	3.47	(2.07 - 4.89)	3.18	(2.58 - 3.57)
Klebsiella spp.	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)	1.10	(1.10 - 1.10)
Non-coliform Gram-negative	5.77	(5.31 - 6.31)	5.68	(5.17 - 6.04)	4.83	(4.06 - 5.55)	5.27	(4.98 - 5.70)
Bacillus spp.	6.80	(6.80 - 6.80)	6.80	(6.80 - 6.80)	5.68	(4.86 - 6.49)	6.80	(6.78 - 6.80)

[1]No *Prototheca spp*. colonies were identified in any bedding samples.
[2]SSLO = *Streptococcus spp.* and Strep-like organisms

Table 2.4: Crude prevalence of intramammary infection and odds ratio (OR) estimates from multivariable logistic regression with mixed effects models investigating the association between intramammary infection and bedding material type and sampling period.

	IMI (all pathogens)		NAS-IMI		SSLO-IMI	
	Crude prevalence	OR (95% CI)[a]	Crude prevalence	OR (95% CI)[a]	Crude prevalence	OR (95% CI)[a]
Bedding material type						
Manure solids	19.3%	Ref	12.70%	Ref	2.0%	Ref
New sand	23.9%	1.42 (1.02 - 1.99)	12.50%	0.85 (0.57 - 1.29)	7.1%	3.53 (2.28 - 5.47)
Organic non-manure	22.7%	1.34 (0.93 - 1.91)	10.80%	0.74 (0.48 - 1.14)	6.7%	3.29 (2.10 - 5.16)
Recycled sand	19.0%	1.07 (0.77 - 1.50)	9.20%	0.56 (0.36 - 0.87)	7.3%	3.53 (2.23 - 5.58)
Sampling period						
Summer 2017	23.1%	Ref	13.10%	Ref	5.9%	Ref
Winter 2017-18	18.9%	0.75 (0.66 - 0.84)	9.60%	0.64 (0.54 - 0.76)	5.2%	0.91 (0.71 - 1.17)

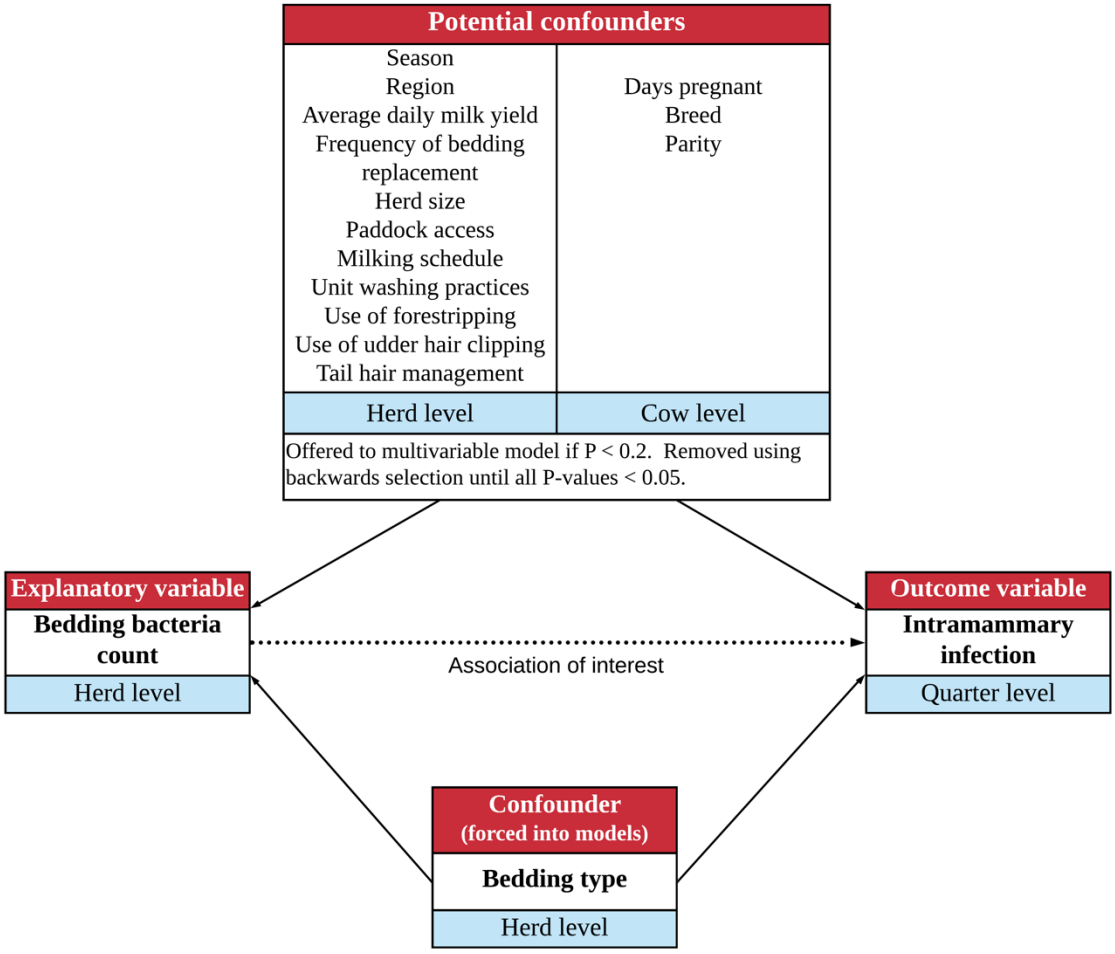

Figure 2.1: Directed acyclic graph illustrating the hypothesized causal relationship between bedding bacteria count and intramammary infection and the model building strategy used to control for variables hypothesized to confound this relationship. The hierarchical structure of the dataset is outlined by describing the measurement level of each variable in blue boxes (i.e. quarter-level, cow-level or herd-level). Random effects terms for cow and herd were used to account for clustering, and confounders were included into multivariable models to adjust for confounding.

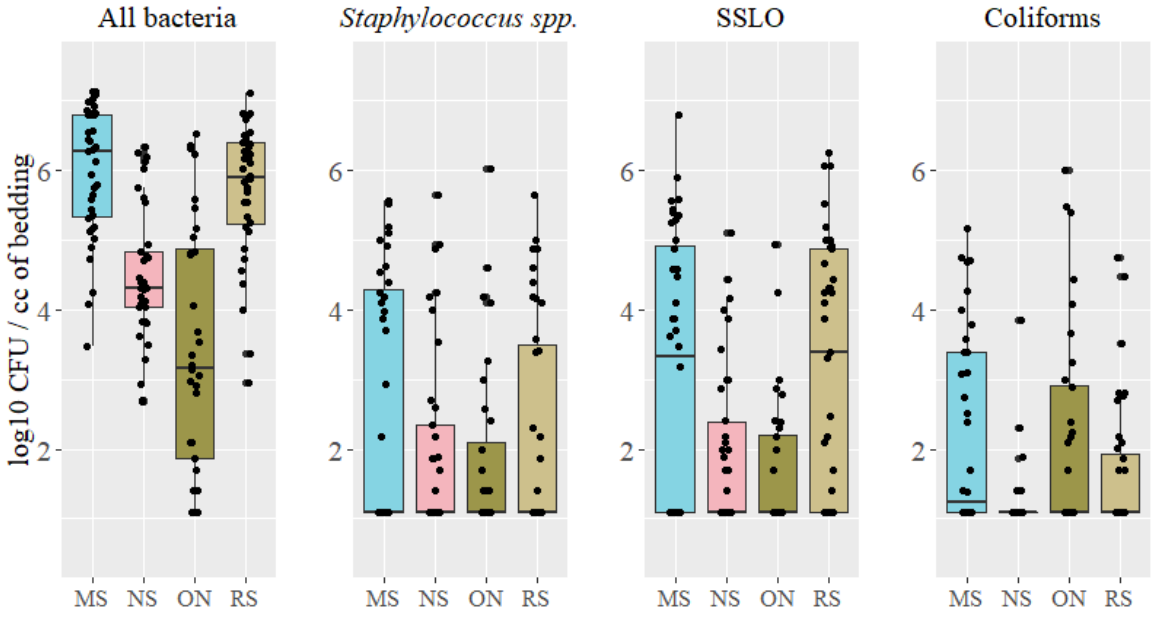

Figure 2.2: Unused bedding. Boxplots showing 25th, 50th (median) and 75th percentiles of bacteria counts in 148 unused bedding samples collected from 76 herds. Black dots indicate data points, which can be seen as clusters at ½ x lower detection limit (1.1 log CFU/cc). The upper detection limit for all counts except 'All bacteria' is 6.8 log CFU / cc. SSLO = Streptococcus and Strep-like organisms, MS = Manure solids (n = 21 herds, 40 samples), NS = New sand (n = 20 herds, 37 samples), ON = Organic non-manure (n = 16 herds, 32 samples), RS = Recycled sand (n = 21 herds, 39 samples). Note that the number of herds does not sum to 76, as some herds switched bedding material type between sampling periods.

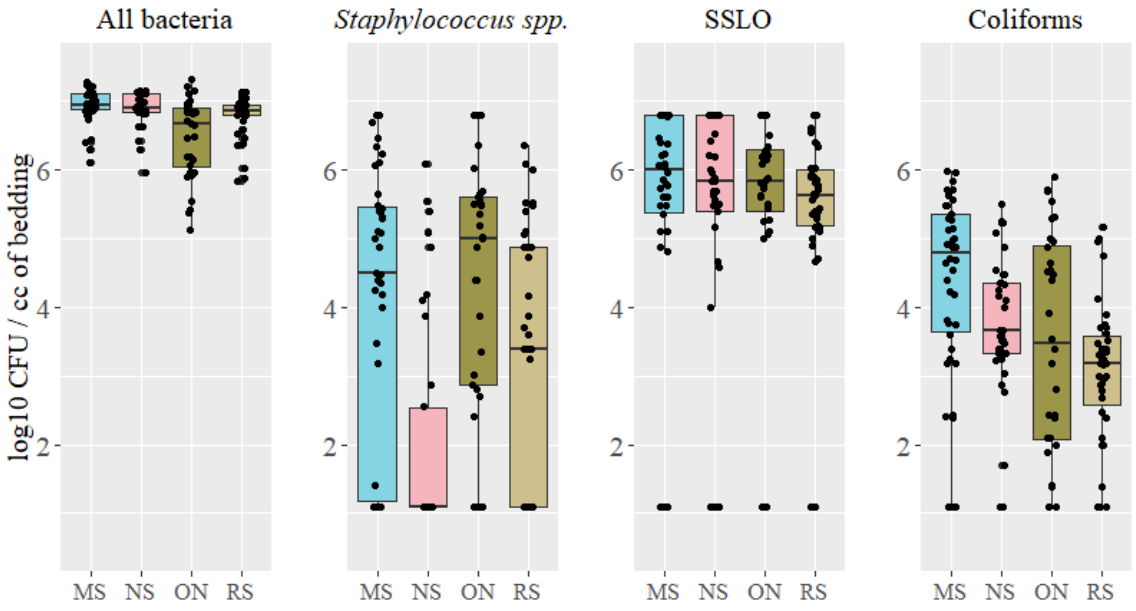

Figure 2.3: Used bedding. Boxplots showing 25th , 50th (median) and 75th percentiles of bacteria counts in 148 used bedding samples collected from 78 herds. Black dots indicate data points, which can be seen as clusters at ½ x lower detection limit (1.1 log CFU/cc). The upper detection limit for all counts except 'All bacteria' is 6.8 log CFU / cc. SSLO = Streptococcus and Strep-like organisms, MS = Manure solids (n = 23 herds, 42 samples), NS = New sand (n = 20 herds, 37 samples), ON = Organic non-manure (n = 16 herds, 32 samples, RS = Recycled sand (n = 21 herds, 39 samples). Note that the number of herds does not sum to 78, as some herds switched bedding material type between sampling periods.

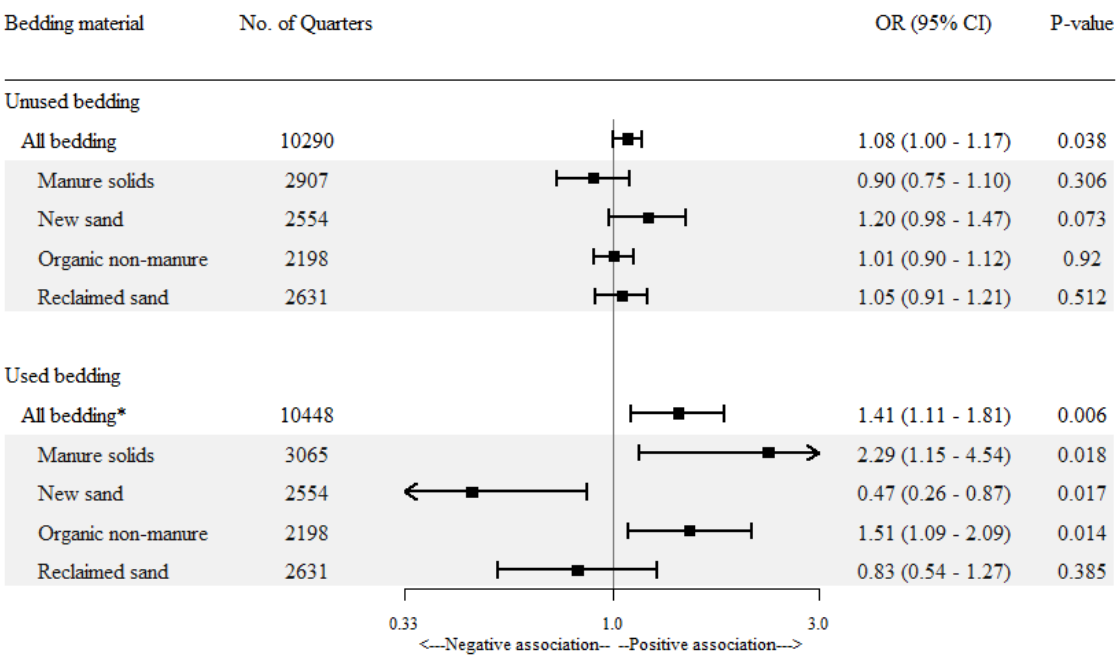

Figure 2.4: ALL-IMI Models. Odds ratio (OR) estimates and 95% confidence intervals (95% CI) from ten multivariable logistic regression with mixed effects models investigating the association between bacteria levels in bedding and intramammary infection (all pathogens). Beta coefficients for covariates are not reported for clarity. *Interaction between bedding bacteria count and bedding type: main effects model reported.

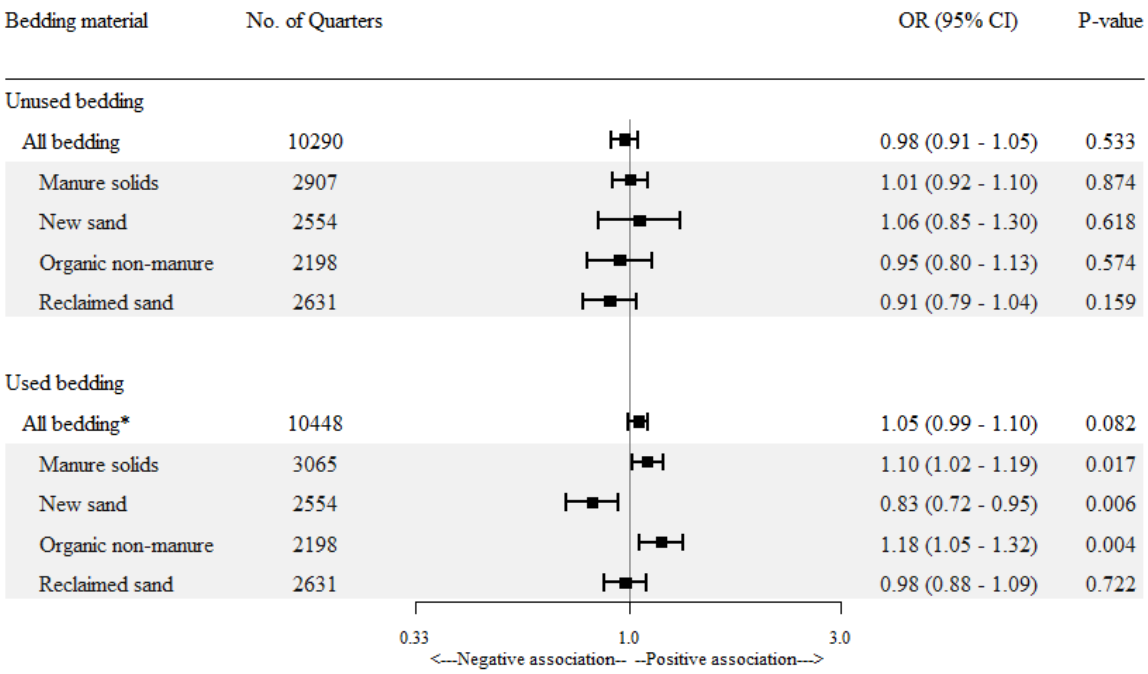

Figure 2.5: NAS-IMI models. Odds ratio (OR) estimates and 95% confidence intervals (95% CI) from ten multivariable logistic regression with mixed effects models investigating the association between bacteria levels in bedding and intramammary infections caused by non-aureus Staphylococcus spp. Beta coefficients for covariates are not reported for clarity. *Interaction between bedding bacteria count and bedding type: main effects model reported.

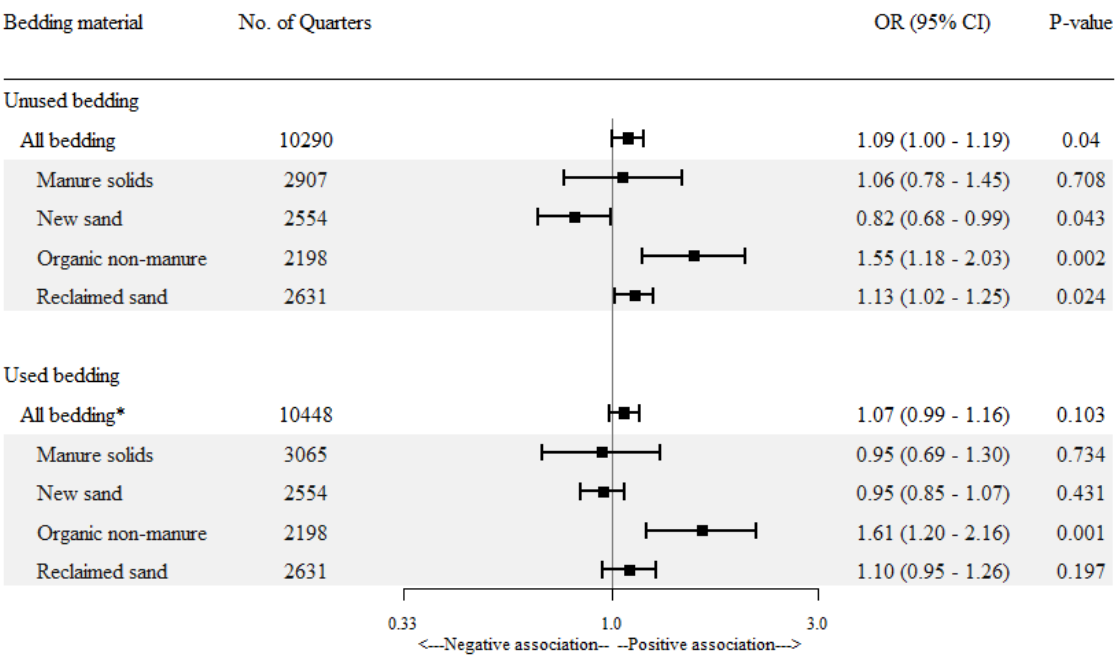

Figure 2.6: SSLO-IMI Models. Odds ratio (OR) estimates and 95% confidence intervals (95% CI) from ten multivariable logistic regression with mixed effects models investigating the association between bacteria levels in bedding and intramammary infections caused by Streptococcus spp. and Strep-like organisms. Beta coefficients for covariates are not reported for clarity. *Interaction between bedding bacteria count and bedding type: main effects model reported.

3 CHAPTER THREE: Randomized Controlled Non-Inferiority Trial Investigating the Effect of Two Selective Dry Cow Therapy Protocols on Antibiotic Use at Dry-Off and Dry Period Intramammary Infection Dynamics

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3.1 Summary

Selective dry cow therapy (**SDCT**) could be used to reduce antibiotic use on commercial dairy farms in the U.S., but is not yet widely adopted, possibly due to concerns about the potential for negative impacts on cow health. The objective of this study was to compare culture- and algorithm-guided SDCT programs to blanket dry cow therapy (**BDCT**) in a multi-site, randomized, natural exposure, non-inferiority trial for the following quarter-level outcomes: antibiotic use at dry-off, dry period intramammary infection (**IMI**) cure risk, dry period new IMI risk, and IMI risk at 1-13 days in milk (**DIM**). Two days before planned dry-off, cows in each of 7 herds were randomly allocated to BDCT ("Blanket"), culture-guided SDCT ("Culture") and algorithm-guided SDCT ("Algorithm"). At dry-off, Blanket cows received an intramammary antibiotic (500mg ceftiofur hydrochloride) in all four quarters. Antibiotic treatments were selectively allocated to quarters of Culture cows by only treating quarters from which aseptically collected milk samples tested positive on the Minnesota Easy® 4Cast® plate after 30-40 hours of incubation. For Algorithm cows, antibiotic treatments were selectively allocated at the cow-level, with all quarters receiving antibiotic treatment if the cow met at least one of the following criteria: 1) any Dairy Herd Improvement Association (**DHIA**) test with a somatic cell count (**SCC**) >

200,000 cells / ml during the current lactation; 2) two or more clinical mastitis cases during the current lactation; and 3) one or more clinical mastitis cases in the 14 days period before enrollment. All quarters of all cows were treated with an internal teat sealant (**ITS**). Intramammary infection status at enrollment and at 1-13 DIM was determined using standard bacteriological methods. The effect of treatment group on dry period IMI cure, dry period new IMI and IMI risk at 1-13 DIM was determined with generalized linear mixed models (logistic), using marginal standardization to derive risk difference (RD) estimates. Quarter-level antibiotic use at dry-off for each group was Blanket (100%), Culture (45%) and Algorithm (45%). The crude dry period IMI cure risk for all quarters was 87.5% (818 / 935), the crude dry period new IMI risk was 20.1% (764 / 3794) and the prevalence of IMI at 1-13 DIM was 23% (961 / 4173). Non-inferiority analysis indicated that culture- and algorithm-guided SDCT approaches performed at least as well as BDCT for dry period IMI cure risk. In addition, the final models indicated that the risk for each of the three IMI measures were similar between all three treatment groups (i.e. RD estimates and 95% confidence intervals all close to zero). Findings from this study indicate that culture- and algorithm-guided SDCT can substantially reduce antibiotic use at dry-off without negatively impacting IMI dynamics.

3.2 Introduction

Control of IMI during the dry period is necessary to optimize udder health in early lactation (Green et al., 2002) and the use of antibiotic treatments at dry-off (dry cow therapy, **DCT**) has been shown to facilitate IMI control by curing existing infections and preventing the establishment of new infections (Halasa et al., 2009a, Halasa et al.,

2009b). At the industry-level, widespread implementation of whole-herd DCT or BDCT, has contributed to the decline in SCC and IMI prevalence, especially of *Staphylococcus aureus* and *Streptococcus agalactiae* in North America, Europe and Australasia. However, BDCT contributes to almost one third of total antibiotic use in lactating cows on non-organic dairy farms in the United States (Pol and Ruegg, 2007). Given that consumers have increasing concerns about antibiotic stewardship in food animal production, there is great interest to increase the adoption of antibiotic-sparing approaches to DCT.

Selective DCT could be used to strategically allocate antibiotic treatments to individual cows or quarters, and thus reduce antibiotic use at dry-off. This can be achieved with rapid culture systems or by using cow records (typically SCC and clinical mastitis events) in algorithms. Field trials conducted in North America and Europe, using different approaches to SDCT, have demonstrated reductions in antibiotic use at dry-off of 58% (Kabera et al., 2019), 48% (McParland et al., 2019), 60% (Vasquez et al., 2018), 21% (Cameron et al., 2014) and 85% (Scherpenzeel et al., 2014). Substantial reductions in antibiotic use at the industry-level has also been observed in European countries following nation-wide bans on BDCT (Vanhoudt et al., 2018). Despite the potential of SDCT to improve antibiotic stewardship in the U.S. dairy industry, only 10% of U.S. herds practice SDCT, with the remaining herds practicing BDCT (80%) or no DCT (10%) (NAHMS, 2014b). This low level of uptake in commercial farms is likely due to perceived risks to cow health and farm profitability, and logistical challenges surrounding the implementation of SDCT programs. Concerns about potential negative health impacts may be in response to the failure of early SDCT approaches, which increased new IMI

risk and IMI prevalence post-calving (Berry and Hillerton, 2002, McDougall, 2010, Scherpenzeel et al., 2014). The failure of these SDCT programs may have been due to the use of screening strategies with insufficient diagnostic sensitivity to detect IMI and/or failure to use a teat sealant to protect against new IMI during the dry period. However, more recent trials of culture- (Cameron et al., 2014, Patel et al., 2017, Kabera et al., 2019) and algorithm-guided (Bradley et al., 2010, Vasquez et al., 2018) SDCT programs, all using teat sealants, found negligible effects on IMI dynamics during the dry period. However, because the aforementioned recent successful SDCT programs conducted in the U.S. enrolled cows from a single herd, multi-herd studies are warranted to explore the generalizability of their findings. In addition to diagnostic accuracy, other considerations affecting test utility include cost, timeliness, and convenience of implementation. Algorithm-guided SDCT programs, which use previously collected SCC data and clinical mastitis history to indirectly predict a cow's risk of infection at dry off (Vasquez, 2018), will be more convenient and less expensive to implement than Culture-guided SDCT programs, which directly detect the presence of IMI at either the quarter or cow-level (Cameron, 2014; Patel, 2017). However, few studies have directly compared between culture- and algorithm-guided SDCT programs (tho Seeth et al., 2017). The objective of this study was to compare culture and algorithm-guided SDCT programs to BDCT in a multi-site, randomized, natural exposure, non-inferiority trial for the following quarter-level outcomes: antibiotic use at dry-off, dry period IMI cure risk, dry period new IMI risk, and IMI risk at 1-13 DIM. We hypothesized that antibiotic use at dry-off could be reduced in commercial dairy herds in the U.S. by implementing either

an algorithm-guided or culture-guided SDCT program, without negatively impacting cow health, as identified using measures of dry period IMI dynamics.

3.3 Materials and Methods

A randomized, controlled, natural exposure study of SDCT in U.S. dairy herds was conducted between May 2018 and April 2019. The Reporting Guidelines for Randomized Controlled Trials for Livestock and Food Safety (REFLECT) (O'Connor et al., 2010) and Reporting of Non Inferiority and Equivalence Randomized Trials Guidelines (Piaggio et al., 2006) were followed in the reporting of this study. Ethics approval was granted by the University of Minnesota Institutional Animal Care and Use Committee (#1801-35489A).

3.3.1 Study Herds

Seven herds were recruited from five states (New York = 2 herds, Minnesota = 1 herd, Wisconsin = 1 herd, Iowa = 1 herd, and California = 2 herds) from May to July, 2018. Herds were selected because they had a working relationship with the University of Minnesota, Cornell University, Iowa State University, DairyExperts Inc. or Dairy Health & Management Services (**DHMS**), had a herd size sufficiently large to dry off 15 cows per week, had an average bulk milk SCC less than 250,000 cells / ml during the previous 12 months, were on a monthly Dairy Herd Improvement Association (**DHIA**) testing schedule (i.e. individual cow SCC and milk weight measurements) and routinely and consistently recorded clinical mastitis and culling events. The bulk tank SCC threshold was used as an inclusion criteria as it has been suggested by mastitis experts that lower bulk milk SCC herds are more likely to benefit from SDCT (Bradley et al., 2018).

3.3.2 Cow Enrollment Visit (2 days prior to dry-off)

Phases of cow enrollment and dry-off treatments are summarized in Figure 3.1. Study technicians visited herds each week to enroll cows two days prior to the planned dry-off date. Cows were eligible for enrollment if they had 4 functional quarters, an expected dry period length of 30 to 90 days, no recent antibiotic or anti-inflammatory treatment (within 14 d), no clinical mastitis (i.e. no visible abnormalities present in foremilk or heat, erythema or pain detected upon palpation of the udder) and not a high locomotion score (must be < 4 on the 5 point scoring system proposed by Sprecher et al. (1997)) or in poor body condition (must be ≥ 1 on the 5 point dairy scoring system proposed by Edmonson et al. (1989)). Following enrollment, duplicate, aseptic quarter-milk samples were collected from enrolled cows according to NMC guidelines (NMC, 2017). Briefly, after milking staff performed their usual pre-milking teat disinfection routine, study technicians, who were wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs, discarded three squirts of foremilk and sampled approximately 20-30 ml of milk into two sterile 60-ml vials. Samples were immediately chilled on ice and transported back to the site laboratory.

Cows were block-randomized (block size = 18, number of unique blocks = 11) to one of three treatment groups by study technicians; Blanket DCT ("Blanket"), Culture-based SDCT ("Culture"), and Algorithm-based SDCT ("Algorithm"). The sequence of treatment groups within each block was determined using a random number generator in Microsoft Excel (Redmond, WA), which was printed onto the set of enrollment forms which were used at all sites. Randomization was stratified by herd and enrollment date.

Following enrollment, cows were milked by farm staff according to each farm's usual routine until the day of dry-off. On two farms (Herds 5 and 6), cows were milked once daily until dry-off. For Herd 6, a commercial external teat sealant product (T-Hexx Dry, Huvepharma) was applied to the teats after each milking. Other herds were milked twice or three-times daily until dry-off.

3.3.3 Rapid culture

Upon arrival back to the site laboratory, one of each duplicate of the quarter milk samples from cows in the Culture group were plated onto the Minnesota Easy® 4Cast® plate using sterile cotton-tip swabs and incubated at 37 ± 2°C for 30-40hrs. All milk samples, including those used for inoculating the rapid culture plates, were stored at -20°C for later laboratory analysis as described below. Minnesota Easy® 4Cast® plates were read by a study technician the morning of the dry-off visit, and quarter-level results recorded as 'growth' or 'no growth'. Samples with growth patterns suggestive of contamination (i.e. numerous independent isolates) were classified as growth.

3.3.4 Dry-off Visit

Dry-off was conducted two days after enrollment. All quarters of Blanket cows were treated with an intramammary antibiotic (500mg ceftiofur hydrochloride, SPECTRAMAST® DC, Zoetis. Parsippany, NJ). In Culture cows, individual quarters were treated with antibiotics if any growth was observed on the Minnesota Easy® 4Cast® plate. Algorithm cows had all 4 quarters treated with antibiotics if they met any of the following criteria for treatment: ≥2 cases of clinical mastitis during lactation, ≥1 case of clinical mastitis during the 14 day period preceding dry-off or any DHIA test with

a SCC > 200,000 cells/ml during lactation. This algorithm was slightly modified from an earlier algorithm described by Vasquez et al. (2018), and was intended to identify cows most likely infected with major pathogens by using both SCC and clinical mastitis records for the current lactation. All quarters of all cows were infused with an ITS containing bismuth subnitrate (ORBESEAL®, Zoetis. Parsippany, NJ). Antibiotic and ITS infusions were administered after the final milking in the following fashion: study technicians, who were wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs for at least 5 seconds before the antibiotic treatment was infused into the mammary gland and again before ITS was infused into the teat cistern. A partial insertion technique was used for both antibiotic and ITS treatments. Cows that received the incorrect antibiotic treatments were either excluded or reassigned to the BDCT group. Consequently, analysis was conducted on an 'as-treated' basis, which is preferable to 'intention to treat' in non-inferiority designs (Piaggio et al., 2006). All procedures were conducted by trained technicians.

3.3.5 Follow-up During the Dry Period and Post-Calving

Clinical diseases during the dry period and early lactation were detected and recorded by farm staff, who were masked to treatment status of cows and laboratory culture results. On two farms, the herd manager was granted access to antibiotic treatment records, which could have allowed them to indirectly determine treatment status of cows. A companion manuscript reports clinical mastitis events, culling and death events, SCC and milk yield outcomes during the dry period and following lactation (Rowe et al., In-Press-a) (see chapter 4). Duplicate, aseptic quarter milk samples were collected by a study technician at 1-13 DIM using the same methods described for the enrollment sampling.

Samples were immediately chilled on ice and transported back to the site laboratory for testing.

3.3.6 Microbiological Culture of Milk Samples

Milk samples were stored at -20°C for 1-7 days before being cultured. The IMI status of each quarter at enrollment and 1-13 DIM was determined using standard bacteriological methods. Milk culture was completed at the regional lab (Minnesota, New York, Iowa, California) using culture methods that were standardized across the four labs. Laboratory staff were masked to the treatment group. On the day of testing, milk samples were thawed at room temperature, homogenized by gentle inversion and plated onto Trypticase Soy Agar with 5% Sheep Blood. Agar plates were inoculated with one loop-full (approximately 10µl) of sample, using disposable plastic loops and incubated in aerobic conditions and at 37 ± 2°C for 42-48 hours. Only one sample from each quarter was cultured, unless the first sample was contaminated. Samples were classified as contaminated if three or more distinct microbial isolates were recovered. This meant that for Culture cows, the sample used for inoculating the Minnesota Easy® 4Cast® plate was used a second time for laboratory culture when the other sample was contaminated. Care was taken to avoid contamination of milk samples when they were used for testing. At the regional labs in Minnesota, New York and Iowa, all isolates were identified using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Microflex; Bruker Daltonics Inc, Billerica, MA). Peaks produced by each isolate were analyzed by the MALDI-TOF Biotyper reference library. The confidence level for each diagnosis reported by the software was used in the following fashion: >2.0,

species level diagnosis recorded; 1.8 – 2, genus level diagnosis recorded; <1.8, MALDI-TOF diagnosis not recorded and traditional identification methods used. Traditional identification methods included colony morphology, catalase reaction, Gram-stain and cytology. Because the California lab did not have MALDI-TOF available for isolate identification, isolates identified here were stored in glycerol vials at -80°C before submitting them to the Minnesota laboratory. Isolates sent to Minnesota were cultured on Trypticase Soy Agar with 5% Sheep Blood in aerobic conditions and incubated at 37 ± 2°C for 24 hours before being identified using the same methods described earlier. If the culture from a glycerol vial failed to yield a single isolate (i.e. no growth or two or more isolates), then the original milk sample was cultured again in California and the isolate was resubmitted. If no pathogen was isolated on the second milk culture at the California laboratory, then the quarter was excluded from analysis. To improve the specificity of IMI classification (i.e. reduce false positives), non-aureus *Staphylococcus spp*. (**NAS**) isolates with less than 2 colonies (<200 CFU/ml) and *Bacillus spp*. isolates with less than 5 colonies (<500 CFU/ml) were reclassified as 'no growth' and the quarter considered uninfected (Dohoo et al., 2011b). This adjustment was made because poor specificity is a more potent source of biased measures of association than poor sensitivity (Haine et al., 2018).

3.3.7 Statistical Analysis

3.3.7.1 Sample Size Calculation.

Sample size was calculated to enable non-inferiority analysis of dry period IMI cure risk. We assumed that 20% of quarters would be infected at dry-off and that the dry period

cure risk in Blanket quarters would be 88% (Arruda et al., 2013, Johnson et al., 2016). The margin of non-inferiority, alpha and power were set at -10%, 2.5% and 80% respectively. The sample size required, according to these assumptions, was multiplied by 1.3 to account for clustering within the data and to account for missing cure statuses in 10% of quarters, due to contamination of milk samples or failure to collect a milk sample within 13d of calving.

3.3.7.2 Variable Management.

Cow- and quarter-level enrollment and dry-off treatment records, herd demographic information and laboratory findings were recorded in spreadsheets (Google Sheets; Mountain View, CA). Disease events (clinical mastitis, culling and death) and SCC and milk yield records from monthly DHIA testing were extracted from electronic farm records. Data were imported into the R Statistical Programming Environment (R Core Team, 2018) for merging and cleaning. Analysis was conducted in R and STATA (version 15; StataCorp, College Station, TX). The analysis log can be found at https://samrowe101.github.io/SDCT-2019/QTRoutcomes.html/. No imputation methods were used for predictor variables, as very few data were missing. Normality of continuous variables was assessed by visualizing normal quantile-quantile plots. All SCC measures (SCC peak during previous lactation and SCC at last DHIA test) were $\log_e$ transformed for analysis. Cows and quarters were retrospectively excluded from analysis if they failed to meet inclusion criteria. Consequently, cows with a dry period outside of the 30 to 90 day range, including cows that failed to calve or were culled during the dry period, were excluded from analysis. Furthermore, treatment records were used to retrospectively exclude quarters from cows with antibiotic treatment during the 14 day

period before milk sampling (at enrollment or post-calving). Quarters without a determined IMI status, mostly due to contamination of milk samples, were not imputed and thus excluded from analysis (Dohoo et al., 2016).

3.3.7.3 Effect of Selective Dry Cow Therapy on Quarter-Level Dry Period Intramammary Infection Dynamics.

Intramammary infection status at enrollment and post-calving was used to determine dry period infection dynamics, including IMI cure and new IMI. Consequently, quarters missing an IMI status at one or both periods were not assigned a value for dry period IMI cure or new IMI. Only quarters with an IMI at the enrollment sample were considered at risk for a dry period IMI cure. Dry period IMI cure cases were defined as a quarter with a species-level IMI present at enrollment that was not isolated in the post-calving sample. All quarters were considered at risk for developing a dry period new IMI. Dry period new IMI cases were defined as a quarter with a species-level IMI at calving that was not originally present in the enrollment sample. For example, a quarter with a *Staphylococcus chromogenes* IMI at enrollment and no growth at 1-13 DIM would be coded as a dry period IMI cure = 1, and dry period new IMI = 0. A quarter with a *Staphylococcus chromogenes* IMI at enrollment and a *Staphylococcus haemolyticus* IMI at 1-13 DIM would be coded as a dry period IMI cure = 1 and a dry period new IMI = 1. Isolates were matched at the genus-level if the species was not known. For example, a quarter with a *Staphylococcus chromogenes* IMI at enrollment and a *Staphylococcus sp.* IMI at 1-13 DIM would be coded as a dry period IMI cure = 0, and dry period new IMI = 0.

Unconditional relative risk estimates for the relationship between explanatory variables of interest and IMI at enrollment, IMI at 1-13 DIM, dry period cure risk and dry period new IMI risk were calculated using generalized linear models (binomial family, log link; 'log-binomial model'). Potential cow-level confounders investigated included: age at enrollment (months), parity at enrollment (1, 2, ≥3), milk yield (kg) and $\log_e$ SCC (log cells x 10^3/mL) at the most recent herd test prior to enrollment, peak $\log_e$ SCC during the lactation of enrollment, any clinical mastitis events during the lactation of enrollment (dichotomous), DIM at dry-off (per 10 d) and DIM at post-calving milk sample (d). In addition, quarter-level IMI at enrollment (dichotomous) was considered a potential confounder for the relationship between treatment group and dry period new IMI and IMI risk post calving.

The final multivariable model for the effect of treatment group on quarter-level, IMI outcomes (dry period cure risk, dry period new IMI risk, IMI risk at 1-13 DIM) was determined using generalized linear mixed models (binomial family, logit link; i.e. logistic regression) using the following model building strategy: A directed acyclic graph (DAG) was drawn for each model to identify potential confounders. Correlations between potential confounders were determined using Pearson's correlation coefficient and Kendall's Tau for normally and non-normally distributed continuous variables respectively. Highly correlated variables (> 0.7) were not offered to the same model, with the more suitable variable chosen based on missing values, reliability of measurement or biological plausibility. Potential confounders were simultaneously offered to the initial model. Following this, biologically plausible effect measure modification on the

multiplicative scale was evaluated by fitting interaction terms as fixed effects. For interaction terms with Wald tests at $P < 0.05$, effect estimates were stratified by the effect-modifying variable and if biologically relevant, were included in the final model. Interaction terms were removed from the model if they had Wald tests at $P > 0.05$ or if they did not add subjective explanatory value to the model. Following this, covariates (potential confounders) were removed from the model one at a time, and replaced back into the model if removal changed the effect estimate by more than 10% (Greenland and Pearce, 2015). All models included random intercepts for cow and herd to account for the clustering of quarters within cows, and cows within herds.

Risk difference estimates (**RD**) for the effect of treatment on quarter-level dry period IMI dynamics were derived from the final logistic regression model using the margins command in Stata, a process called 'marginal standardization' (Muller and MacLehose, 2014). A non-inferiority hypothesis test was used to evaluate the effect of SDCT on dry period IMI cure risk, using the confidence interval approach, with an *a priori* margin of non-inferiority specified at -10%. Therefore, the null hypothesis was that cure risk in either SDCT group (Culture or Algorithm) was at least 10% lower than in BDCT quarters. The two-sided, 95% confidence interval for the risk difference was used to conduct the hypothesis test, such that if the lower limit of the confidence interval was greater (i.e. 'better') than the *a priori* margin of non-inferiority, then the experimental group (either SDCT group) would be considered non-inferior to or 'at least as good' as the positive control group (BDCT). We chose to only conduct a non-inferiority test for dry period IMI cures because it was the primary outcome of interest in our study. Additionally, because the original sample size calculations were based on using a non-

inferiority test for dry period IMI cures, it is possible that statistical power would not be sufficient to enable non-inferiority analysis for other outcomes. No superiority hypothesis tests were conducted to evaluate the effect of SDCT group on measures of udder health (Wasserstein and Lazar, 2016).

3.4 Results

3.4.1 Enrollment

Demographic information about the 7 study herds can be found in Table 3.1. All herds were predominantly Holstein cows, except for herd 3, which was predominantly Holstein-Jersey cross. Herd size ranged from 850 to 5700 milking cows and bulk milk SCC prior to enrollment ranged from 90,000 to 230,000 cells / ml. All herds routinely practiced pre- and post-milking teat disinfection as part of their milking routine and used a registered *E. coli* vaccine as part of their mastitis control strategy. In five herds, lactating cows were managed in freestall barns with sand (n = 3) or recycled manure solids (n = 2) bedding. The remaining two herds used a combination of freestalls and composted bedded packs. The dry cow housing facilities used were deep-bedded sand freestalls (n = 4 herds), recycled manure dry-lots (n = 2) and composted bedded pack (n = 1). At enrollment, cows were randomly allocated to Blanket (n = 429, 1716 quarters), Culture (n = 432, 1728 quarters) and Algorithm (n = 414, 1656 quarters) groups. Demographic information about the enrolled cows in each treatment group can be found in Table 3.2. Median age at enrollment (skewed distribution) was 45 months (interquartile range [**IQR**] = 34 – 56), mean DIM at dry-off (normally distributed) was 325 (SD = 46) and mean milk yield at the most recent DHIA test prior to dry-off

(normally distributed) was 27.3 kg (SD = 8.7). The proportion of cows of parity 1, 2 and ≥3 at enrollment was 42%, 30% and 28% and the proportion of cows at enrollment that had experienced at least one clinical mastitis event during the lactation of enrollment was 14%.

3.4.2 Losses to Follow-Up

Losses to follow-up at the cow- and quarter-level during each phase of the study are outlined in Figure 3.2. Three enrolled cows were retrospectively excluded because of recent (<14d) antibiotic treatments before enrollment. Two cows from the Culture group were reassigned to the Blanket group due to study technicians accidentally treating quarters that did not have bacterial growth on the Minnesota Easy® 4Cast® plate. After cows were reassigned, they had all four quarters treated with antibiotics, regardless of culture results.

At dry-off (2 days after enrollment), 29 cows were removed from the study by study technicians because they did not meet the inclusion criteria, mostly due to being found non-pregnant and thus unable to have a dry period less than 90 days. Consequently, the number of cows dried off in each group was Blanket (n = 417), Culture (n = 422) and Algorithm (n = 404). Demographics for study herds and treatment groups at this stage of enrollment are compared in Table 3.1 and Table 3.2 respectively. During the dry period, 11 cows died or were culled from the Blanket (n = 5), Culture (n = 1) and Algorithm (n = 5) groups. Of these, three deaths (2 x Blanket and 1 x Algorithm) occurred in the 14 day period after dry-off. The remaining deaths or culls occurred 21 to 77 days after dry-off. No mastitis cases were recorded for culled or dead animals during the dry period. An additional 17 and 4 cows were retrospectively excluded from the study, because of short

(<30 d) or long (>90 d) dry periods, respectively. In total, 22, 22 and 20 cows from Blanket, Culture and Algorithm groups were lost to follow-up between enrollment and calving, respectively. An additional 35 cows (Blanket = 15, Culture = 12, Algorithm = 8) were excluded from analysis, because of recent (<14 d) antibiotic treatment prior to milk sampling following calving. Six of these cows had been treated for mastitis. The remaining antibiotic treatments were for reproductive disorders, trauma and lameness. Quarters from an additional 65 cows (Blanket = 19, Culture = 19, Algorithm = 27) were excluded due to failure to collect a milk sample within 13 days of calving. An additional 462 (Blanket = 172 / 1716, Culture = 150 / 1728, Algorithm = 140 / 1656) and 271 (Blanket = 100 / 1716, Culture = 90 / 1728, Algorithm = 81 / 1656) quarters were contaminated at the enrollment and post-calving samplings, respectively. Consequently, the number of quarters considered at risk of having an IMI at 1-13 DIM for each group were Blanket (n = 1392), Culture (n = 1426) and Algorithm (n = 1355).

3.4.3 Antibiotic Use

Antibiotic use at the quarter-level for each treatment group were Blanket (1668 / 1668; 100%), Culture (752 / 1688; 44.5%) and Algorithm (724 / 1616; 44.8%) when considering all cows that were dried-off as part of the trial. The proportion of quarters treated per farm ranged from 31.9 to 61.8% for Culture quarters and 18.9% to 68.4% in Algorithm quarters.

3.4.4 Intramammary Infection at Enrollment and at 1 to 13d Days in Milk

The quarter-level prevalence of IMI at enrollment was 25.4% (1078 / 4242; Table 3.3). Of the 1144 cows with a valid culture result at dry-off, 670 (59%) had at least one

infected quarter. Of the cows with at least one infected quarter at enrollment and a full set of culture results (i.e. none excluded for contamination, n = 519), the number of quarters infected per cow were one (275, 53.0%), two (159, 30.6%), three (67, 12.9%) and four (18, 3.5%). The quarter-level prevalence of IMI in Blanket, Culture and Algorithm groups were 25.1% (350 / 1396), 25.0% (360 / 1442) and 25.3% (355 / 1404). The overall quarter-level prevalence of IMI caused by pathogen groups were NAS (14.8%), *Staphylococcus aureus* (0.3%), *Streptococcus* and Strep-like organisms (**SSLO**) (2.2%), other Gram-positive bacteria (7.9%), Gram-negative bacteria (1.5%) and other pathogens (0.1%). Note that these prevalences do not sum to the overall prevalence of IMI (25.4%) because some quarters were infected with two pathogens (n = 91). The most common bacterial species was *Staphylococcus chromogenes*, which infected 8.0% of quarters. Other common causes of IMI at enrollment included *Staphylococcus spp.* (4.0%), *Corynebacterium sp.* (4.0%), *Micrococcus sp.* (1.5%), *Staphylococcus haemolyticus* (1.1%), and *Bacillus sp.* (0.9%). *Staphylococcus aureus* (0.3%), *Streptococcus uberis* (0.1%) and coliforms (0.6%) were uncommon causes of IMI.

The quarter-level prevalence of IMI at 1-13 DIM was 23.0% (961 / 4173; Table 3.4). The prevalences were similar in Blanket (22.8%, 317 / 1392), Culture (23.9%, 341 / 1426) and Algorithm quarters (22.4%, 303 / 1355). Unconditional RR estimates for various predictors of IMI at enrollment and at 1-13 DIM are reported in Table 3.5. The final model for IMI risk at 1-13 DIM is shown in Table 3.6. Adjusted risks and RD estimates for IMI at 1-13 DIM were Blanket (17.2%), Culture (17.5%, RD = +0.3%, 95% CI: -2.4, 3.1%) and Algorithm (16.7%, RD = -0.5%, 95% CI: -3.2, 2.3%). The intraclass correlation coefficients for clustering of quarters within cows and cows within herds were

0.31 and 0.16. The overall quarter-level prevalence of IMI caused by pathogen groups were NAS (13.0%), *Staphylococcus aureus* (0.1%), SSLO (1.4%), other Gram-positive bacteria (7.9%), Gram-negative bacteria (1.9%) and other pathogens (0.3%). Note that these prevalences do not sum to the overall prevalence of IMI (23.0%) because some quarters were infected with two pathogens (n = 107). Like the samples collected at dry-off, the most common bacterial species was *Staphylococcus chromogenes*, which infected 4.6% of quarters. Other common causes of IMI included *Staphylococcus sp.* (4.7%), *Staphylococcus sciuri* (3.1%), *Bacillus sp.* (3.1%), and *Corynebacterium sp.* (2.5%).

3.4.5 Effect of Selective Dry Cow Therapy on dry period IMI Cure and New IMI risk

Unconditional RR estimates for various predictors of dry period IMI cure and new IMI risk are reported in Table 3.5. The crude dry period IMI cure risk for all quarters was 87.5% (818 / 935), which was similar in Blanket (263 / 303, 86.8%), Culture (288 / 329, 87.5%) and Algorithm (267 / 303, 88.1%) treatment groups. The final models evaluating the effects of treatment group on dry period IMI cure risk and new IMI are shown in Table 3.6. Adjusted cure risks and RD estimates were Blanket (89.7%), Culture (89.9%, RD = +0.2%, 95% CI: -4.4, 4.8%) and Algorithm (90.6%, RD = +0.9%, 95% CI: -3.7, 5.5%). The intraclass correlation coefficients for clustering of quarters within cows and cows within herds were 0.31 and 0.04. The lower limits of the RD two-sided 95% confidence intervals for Culture (-4.4%) and Algorithm (-3.7%) quarters did not cross the *a priori* margin of non-inferiority (-10% units), indicating that both SDCT approaches were non-inferior to, or ‘at least as good’ as BDCT (Figure 3.3). The crude dry period New IMI risk for all quarters was 764 / 3794 (20.1%), which was similar in Blanket (246

/ 1255, 19.6%), Culture (272 / 1298, 21.0%) and Algorithm (246 / 1241, 19.8%) treatment groups. Adjusted new IMI risks and RD estimates were Blanket (14.7%), Culture (15.2%, RD = +0.5%, 95% CI: -2.2, 3.2%) and Algorithm (14.7%, RD = +0.0%, 95% CI: -2.6, 2.7%) quarters. The intraclass correlation coefficients for clustering of quarters within cows and cows within herds were 0.28 and 0.15.

3.5 Discussion

3.5.1 Selective Dry Cow Therapy Reduced Quarter-level Antibiotic Use at Dry-off by 55%

This study has demonstrated that quarter-level antibiotic use at dry-off can be substantially reduced using culture or algorithm-guided SDCT. The observed reduction of 55% is consistent with other recent studies using similar approaches (Patel et al., 2017, Vasquez et al., 2018, Kabera et al., 2019, McParland et al., 2019). Although the reduction was the same in Culture and Algorithm quarters overall, they were rarely the same within a single herd. Furthermore, there was considerable among-herd variation in quarter-level antibiotic reductions, with wide ranges for both Culture (32 – 68%) and Algorithm (19 – 68%) groups. This among-herd variation is likely to be influenced by a number of factors, including the prevalence IMI and subclinical mastitis, incidence of clinical mastitis and contamination risk when sampling milk for rapid culture. The observation that culture- and algorithm-guided SDCT had equivalent reductions in inconsistent with a recent trial in Germany that found reductions of 23% and 55% for culture- and algorithm-guided SDCT (tho Seeth et al., 2017).

3.5.2 Selective Dry Cow Therapy Had No Marked Negative Impacts on Quarter-level Measures of Udder Health

This study found that Culture and Algorithm quarters had very similar risks to Blanket quarters for dry period IMI cure, dry period new IMI and IMI at 1-13 DIM. The small and relatively precise (narrow 95% confidence intervals) risk difference estimates indicate that under the conditions of this trial, either SDCT approach is unlikely to cause marked impacts on dry period IMI dynamics.

3.5.2.1 Culture-guided Selective Dry Cow Therapy

The success of culture-guided SDCT in this trial is consistent with recent studies conducted in Canada using 3M™ Petrifilm™ (Cameron et al., 2014, Kabera et al., 2019) and a recent pilot study using the Minnesota Easy® 4Cast® plate (Patel et al., 2017). In contrast, older field trials found that withholding antibiotic treatment from cows or quarters identified to be uninfected using laboratory culture resulted in increased dry period new IMI risk (Browning et al., 1990, Berry and Hillerton, 2002). One clear distinction (among others) between the successful and failed programs was that ITS were used in the successful programs, which is consistent with numerous studies that have consistently shown ITS to substantially reduce dry period new IMI risk (Rabiee and Lean, 2013). Our study is the first multi-herd trial to report outcomes for quarter-level, culture-guided SDCT using the Minnesota Easy® 4Cast® plate. Prior to our study, culture-guided SDCT had only been evaluated using rapid culture results at the cow-level (Cameron et al., 2014, tho Seeth et al., 2017). The main advantage of treating at the quarter-level is that it might allow for greater reductions in antibiotic use at dry-off. For example, the reduction in quarter-level use in this study would have been only 23% if

treatment had been allocated at the cow-level, compared to the 55% that was observed when allocating at the quarter-level. This is because most cows (59%) identified by laboratory culture to have an IMI were infected in a single quarter. However, incorporating all quarters into a single, cow-level result may have some advantages. This is because quarters within the same cow are not truly independent of one-another, which is evidenced in our study by the high ICC for the three reported measures of dry period IMI dynamics shown in Table 3.6 (range 0.29 – 0.31). Consequently, the presence of an IMI in one quarter could be considered as sufficient evidence to treat the remaining quarters of the same cow. Interpreting culture results within the same cow in parallel (i.e. cow-level), could help to increase sensitivity, which has been shown to be low for certain pathogens. This is supported by the findings of a multi-herd trial of laboratory culture-guided SDCT in Australia, which found that quarter-level treatment (as was done in this study) resulted in a higher dry period new IMI risk (6.4%) than cow-level SDCT (3.9%) and BDCT (2.6%) (Browning et al., 1994). In that study, the predominant cause of new IMI was *Staphylococcus aureus*, which typically spreads between quarters during lactation, and not during the dry period. It is therefore possible that many of the 'new infection' cases in that study were actually persistent infections that were not identified as infected at the dry-off sample due to failure of the screening system. Such a failure seems probable, given that *Staphylococcus aureus* is intermittently shed in milk of infected quarters, with one study estimating the sensitivity of a single milk sample being 75% (Sears et al., 1990). The prevalence of *Staphylococcus aureus* IMI has declined significantly since the time of the Browning et al. (1994) study (Rowe et al., 2019), which may explain why quarter-level culture-guided SDCT performed as well as BDCT

in our study and others (Patel et al., 2017, Kabera et al., 2019). In summary, our research indicates that quarter-level SDCT can be implemented without negative impacts on IMI dynamics. However, this approach may not be appropriate in situations when the IMI at dry-off are caused by pathogens that are difficult to detect (i.e. lower sensitivity), behave contagiously, and have a significant impact on cow health if they escape treatment at dry-off (eg. *Staphylococcus aureus, Streptococcus agalactiae*). This requires further investigation.

3.5.2.2 Algorithm-guided Selective Dry Cow Therapy.

The effect of algorithm-guided SDCT on IMI dynamics is consistent with some previous studies, which found negligible impacts on IMI dynamics (Bradley et al., 2010, Vasquez et al., 2018). However, other SDCT studies using an algorithm-based approach have found clear deleterious effects (McDougall, 2010, Scherpenzeel et al., 2014). One clear distinction between the programs was the use of teat sealants in the successful programs. Another potential source of variability in responses to algorithm-guided SDCT is the algorithm itself. Many algorithms have been recommended by mastitis experts (Bradley et al., 2018, Gohary and McDougall, 2018, Vasquez et al., 2018), which use as few data points as possible (typically only using SCC and clinical mastitis data), in order to facilitate implementation under farm conditions. In the study by Scherpenzeel et al. (2014), the SCC component of their algorithm only included the most recent SCC measurement prior to dry-off. Our algorithm, and others (Bradley et al., 2010, Vasquez et al., 2018) interpreted multiple SCC measurements in parallel, which is expected to increase sensitivity. However, the unsuccessful algorithm protocol used by McDougall

(2010) employed parallel interpretation of bi-monthly SCC measurements from the entire lactation and more conservative SCC (150,000 cells/ml) and clinical mastitis (≥ 1 case) thresholds than in our study (200,000 cells/ml and ≥ 1 case respectively), suggesting that the lack of ITS used in that study was the likely reason for the program failure, and not accuracy of the algorithm. To our knowledge, no algorithm-guided SDCT trials have compared effects of different SCC cut points on measures of udder health. However, many algorithms with different SCC thresholds have been evaluated in their ability to predict IMI at dry-off (Pantoja et al., 2009, Gohary and McDougall, 2018, Lipkens et al., 2019). A recent, multi-herd study in Belgium concluded that a single algorithm was unlikely to be highly accurate for all cows, and that other variables, such as herd-level estimates of subclinical mastitis prevalence, cow milk yield and parity should be incorporated into more sophisticated algorithms. The test characteristics of the algorithm used in this study, along with the Minnesota Easy® 4Cast® plate will be compared in a separate report. One limitation for the evaluation of the algorithm in this study was the inclusion criteria, which excluded cows with recent antibiotic treatments (< 14d prior to dry-off) from the study. Unfortunately, excluding such cows (n = 3) rendered part of the algorithm (clinical mastitis within 14d of dry-off) redundant. However, we believe that it is highly unlikely that the exclusion of such a small number of cows from the study would have impacted the validity of our results.

3.5.3 Practical Considerations for SDCT Approaches Used in this Study

This is the first study to directly compare antibiotic use and subsequent quarter health for SDCT programs using quarter-level culture and a cow-level algorithm. Given that both

approaches yielded similar antibiotic reductions and health outcomes, it is likely that producers could use other factors to guide their decisions around SDCT implementation, such as feasibility and economics. Some potential advantages of culture-guided SDCT is that it does not require herds to conduct monthly DHIA testing or record clinical mastitis during lactation, which currently accounts for approximately only 44% and 27% of herds in the U.S, respectively (NAHMS, 2014a). Furthermore, the use of rapid culture systems may enable farmers to identify cows infected with pathogens of interest for specific interventions (eg. culling *Staphylococcus aureus* cows), though this idea requires further study. One disadvantage of the culture-guided SDCT approach is that quarter-level detection and treatment adds complexity, and thus, the potential for mistakes during the dry-off procedure.

One of the major advantages of algorithm-guided SDCT is that it efficiently utilizes data that is already available on many farms. Furthermore, allocation of treatments at the cow-level is likely to be easier for farm staff to successfully implement than quarter-level treatments. The disadvantages of algorithm-guided SDCT is that it requires the herd to be participating in a DHIA testing program, and be accurately detecting and recording clinical mastitis events into electronic herd records. Furthermore, a number of studies have shown the algorithms are not sensitive predictors of IMI caused by 'minor' pathogens like NAS and *Corynebacterium spp.*, because such IMI often fail to increase SCC above commonly used SCC thresholds (eg. 200,000 cells / ml) (Gohary and McDougall, 2018). However, this potential limitation may actually be an advantage, as it is unclear if quarters infected with minor pathogens benefit from antibiotic treatment. Previous studies have found that NAS IMI at dry-off have a high spontaneous dry period

cure risk (Vasquez et al., 2018) and often have minimal effects on clinical mastitis risk (Green et al., 2002) or milk production in the subsequent lactation (Vanderhaeghen et al., 2014). Given that NAS are the main cause of IMI at dry-off, we believe that more research is needed to investigate the importance of treatment of NAS-infected quarters. The economic impact of each approach is likely to be a potent driver of farmer decisions around SDCT. Currently, economic modelling of DCT approaches under U.S. conditions has not yet been conducted, and therefore, we plan to address this using stochastic modelling methods in a separate study. Economic modelling of BDCT and algorithm-guided SDCT in the Netherlands have found that algorithm-guided SDCT resulted in higher net financial gains than BDCT, despite having some negative effects on cow health (Scherpenzeel et al., 2016, Scherpenzeel et al., 2018).

Regardless of the program selected, implementation of a successful SDCT program will require some consideration of appropriate selection of candidate farms, and careful attention to correctly manage and implement the program. While no formal research exists on the question of farm selection, mastitis experts have suggested that herds free from *Streptococcus agalactiae* and with bulk tank SCC below 250,000 cells / ml, may be more likely to benefit from SDCT (Bradley et al., 2018). In addition, we believe it is important that candidate farms have excellent record management, train farm staff to successfully utilize the culture or algorithm technology, and ensure strict adherence to clean intramammary infusion techniques, particularly when infusing a quarter with ITS alone. We believe that SDCT programs represent an opportunity for the local veterinary practices to offer the necessary services for candidate farms that aren't willing or capable of managing a SDCT program with their own staff. For example, for those farms not

interested or able to set up a successful on-farm culture program, it is possible that in-house rapid culture services could be provided by the local veterinary clinic. Furthermore, trained veterinary technicians could offer related services including aseptic collection of milk samples for culture prior to dry-off, accurate interpretation of culture (or algorithm) results, and clean, correctly applied infusion of the assigned treatments on the day of dry-off.

3.5.4 Prevalence of Intramammary Infection at Dry-off and Antibiotic Choice

The quarter-level prevalence of IMI at enrollment (two days prior to dry-off) was 25%, which is similar to a recent survey of over 10,000 quarters of late lactation cows from 80 herds, in 10 states of the U.S., which found a prevalence of 21% (Rowe et al., 2019). In the current study, most infections were caused by Gram-positive bacteria (1024 of 1078 infected quarters, 95%), which were mostly NAS (629 of 1078 infected quarters, 58%). The most common pathogens were *Staphylococcus chromogenes* (8% of quarters at risk) and *Staphylococcus sp.* (i.e. underdetermined species, 4%). Only 63 of 4242 (1.5%) quarters at risk were infected with Gram-negative bacteria, which accounted for only 5.8% of all infected quarters. This is consistent with the Rowe et al. (2019) study, which found a Gram-negative prevalence of 0.8% (3% of infected quarters). This low prevalence of Gram-negative IMI at dry-off may explain why some recent clinical trials have found no difference in dry period IMI cure and new IMI risk in quarters treated with narrow or broad-spectrum antimicrobials (Arruda et al., 2013, Johnson et al., 2016). Despite the questionable benefit of using broad-spectrum antibiotics at dry-off, we chose to use a broad spectrum antibiotic (500mg ceftiofur hydrochloride) as part of our DCT

protocols, as we wanted to validate our SDCT approaches against a BDCT protocol that is likely to be perceived by veterinarians and producers as being 'comprehensive'. Other successful SDCT protocols have used third (Cameron et al., 2014) and first generation cephalosporin antibiotics (Bradley et al., 2010, Vasquez et al., 2018), which are broad and narrow in their spectrum of activity, respectively. In light of these studies, and those showing similar health outcomes for narrow and broad spectrum antibiotics used within BDCT protocols, we think there is sufficient evidence to suggest that SDCT could be successfully implemented using narrow spectrum antibiotics. However, this requires further investigation.

3.5.5 Intramammary Infection risk at 1-13 Days in Milk and Infection Dynamics over the Dry Period

Non-aureus *Staphylococcus spp.* were the predominant cause of IMI at 1-13 DIM (544 of 961 infected quarters, 57%), which is very similar to the proportion (58%) at enrollment. The predominant NAS was *Staphylococcus chromogenes* (4.6% of quarters at risk) and *Staphylococcus sp*. (4.7%), which were also the predominant causes of IMI at enrollment. The prevalence of *Staphylococcus sciuri* at 1-13 DIM (3.1%), was much higher than the prevalence at dry-off (0.4%), which indicates that the majority of these infections were acquired from the environment during the dry period. This hypothesis is supported by a study by Piessens et al. (2011), which showed that the reservoirs for *Staphylococcus sciuri* IMI were commonly found in the environment, and less often in cow-associated reservoirs. The prevalence of Gram-negative IMI at 1-13 DIM was only 1.9%, which is similar to the prevalence at dry-off (1.5%). This finding is not consistent with other studies that have shown higher prevalences at calving, due to new Gram-negative IMI

acquired during the dry period. For example, a recent observational study of 1816 quarters from 12 European dairy herds found that the prevalence of Gram-negative IMI at dry-off and post-calving were 2.8 and 4.8% respectively (Bradley et al., 2015). In that study, ITS were not used, which may explain why Gram-negative IMI increased over the dry period. The crude dry period IMI cure risk in our study was 87.5%. This number is similar to recent studies that have found IMI cure risks of 90% (Ospina et al., 2016), 89% (Arruda et al., 2013), 85% (Johnson et al., 2016) and 80% (Bradley et al., 2010). The crude dry period new IMI risk in our study was 20.1%, which is similar to recent studies that found new IMI risks of 13% (Arruda et al., 2013), 19% (Johnson et al., 2016) and 30% (Bradley et al., 2010).

3.5.6 Study Strengths and Limitations

3.5.6.1 Study Design.

One considerable strength of this study is the large number of quarters enrolled (~1700 per treatment group), which provided precise RD estimates (i.e. relatively narrow 95% confidence intervals). Furthermore, this is the first SDCT study to use a non-inferiority design, which in our opinion, is more appropriate than superiority designs for showing 'sameness' between groups. However, we only conducted a non-inferiority hypothesis test for dry period IMI cure risk (Figure 3.3), because it was the primary outcome of interest. For dry period new IMI and IMI at 1-13 DIM risk, no non-inferiority hypothesis tests were conducted, and instead, 95% confidence intervals were reported, which also clearly demonstrated that the effect of SDCT on these measures of udder health is likely to be weak or absent.

3.5.6.2 Internal Validity.

Confounding and selection bias are unlikely sources of bias in our study. Our randomization method appeared to be effective, given the balance of herds and demographic details between treatment groups at baseline (Table 3.2). In addition, no covariates were found to change effect estimates by >10% when they were removed from models, indicating that there was little or no measured confounding. There was no evidence for differential loss to follow-up, which included losses during the dry period due to death or culling and for antibiotic treatments in early lactation, indicating that the effect estimates reported in this study are not likely to be biased by drop-out. However the losses (17% overall) did reduce sample size, which would have likely reduced the precision of effect estimates (i.e. widening of 95% confidence intervals).

It is important to consider potential sources of information bias. The outcome variables in this study were based on IMI measurements taken at enrollment and at 1-13 DIM. Intramammary infection dynamics are commonly used as an udder health measure in DCT research, as they are associated with more tangible measures of udder health like clinical mastitis (Green et al., 2002), but can be measured in an objective, consistent way across multiple herds and sites. To achieve this in our study, laboratory staff at each site were instructed to follow a standardized protocol and to use the same culture mediums and equipment (i.e. MALDI-TOF). However, the use of milk culture to identify IMI has some limitations. Firstly, milk culture is not 100% sensitive nor 100% specific. One study found the sensitivity and specificity of a single milk culture was 70% and 90% respectively (Dohoo et al., 2011a), which would lead to misclassification of infected and

uninfected quarters. However, we believe this potential misclassification would have been the same for quarters of all three treatment groups (i.e. 'non-differential'), which in most cases, causes a bias that reduces the estimated effect size (i.e. 'observed' RD are closer to zero than the 'true' RD). This potential source of information bias is important to consider in non-inferiority and equivalence studies, as it can cause investigators to incorrectly conclude that treatment groups are the same. In our study, we proactively took steps to address potential information bias, and thus improve our chances of detecting a difference in IMI dynamics between groups. Firstly, we used CFU thresholds for NAS and *Bacillus spp*. isolates, in order to increase specificity, which has a more profound biasing effect than sensitivity (Haine et al., 2018). We decided this for NAS because they are the most common cause of IMI at dry-off (Rowe et al., 2019) and diagnostic specificity using milk culture from a single sample is worse than for other pathogens (Dohoo et al., 2011b). We used a similar approach for *Bacillus spp*. Use of CFU thresholds has been conducted in some recent SDCT studies (Cameron et al., 2014), but not all (Scherpenzeel et al., 2014). In addition to CFU thresholds, we used MALDI-TOF mass spectrometry in the diagnosis of pathogens, which has been shown to be superior to traditional biochemical testing methods, especially when an expanded database is used (Barreiro et al., 2010, Wanecka et al., 2019), which had been done prior to the study as part of each laboratory's quality control program. Having highly accurate diagnoses is particularly important in determining dry period IMI dynamics. For example, a quarter infected with *Staphylococcus chromogenes* at dry-off and then *Staphylococcus haemolyticus* at calving can be correctly identified using MALDI-TOF as cure = 1 (*Staphylococcus chromogenes*) and new IMI = 1 (*Staphylococcus haemolyticus*). In

contrast, traditional laboratory methods would often classify both of these IMI as coagulase negative *Staphylococcus spp.* (CNS), and the quarter would be incorrectly classified as cure = 0 and new IMI = 0. However, we did find a substantial proportion of NAS isolates in enrollment (27%) and post-calving (36%) milk samples could not be identified at the species level.

3.5.6.3 External Validity.

We believe that our research is relevant to a large proportion of American dairy farmers, because cows were enrolled from seven herds located in the south-west, mid-west, and north-east dairy farming regions of the U.S. However, the herd inclusion criteria used in this study need to be considered when relating our results to individual dairy herds. These criteria included an existing relationship with the University of Minnesota, Cornell University, Iowa State University, DairyExperts Inc. or DHMS, be drying off at least 15 cows per week, have a bulk milk SCC < 250,000 cells/ml, be on a monthly DHIA testing schedule and have high quality clinical mastitis and culling records. Another important consideration is that the dry-off protocols in this trial were conducted by study technicians and not farm staff, which included the collection of milk samples, reading of Minnesota Easy® 4Cast® plates and administration of intramammary treatments. We think it is possible that some herds with poor mastitis control (which could be identified as SCC > 250,000 cells/ml, or poor control of pathogens like *Staphylococcus aureus* and *Streptococcus agalactiae*), or herds that are unable to carefully implement a SDCT program may be at a higher risk of the SDCT-related adverse health events that were observed in other trials (Berry and Hillerton, 2002, Scherpenzeel et al., 2014).

Furthermore, it should be noted that the incidence of clinical mastitis in the lactation prior to enrollment for the cows in this study was 13.8%, which may less than some U.S. dairy herds. The most recent NAHMS survey of U.S. dairy farms (2014b) estimates that 24.8% of cows experience at least one case of clinical mastitis, and that culling and/or death risk for cows with clinical mastitis is 27.2%. Therefore, the proportion of cows that reach dry-off with a history of clinical mastitis may be approximately 18.1% (i.e. 24.8% multiplied by 1 minus 27.2%).

To our knowledge, no randomized controlled trials have evaluated farmer-implemented SDCT protocols. Furthermore, no observational studies have directly evaluated herd-level factors that might modify the effect of SDCT on dry period IMI dynamics. Another important limitation to the generalizability of this study is that cows were followed for a relatively short period of time, which may reduce generalizability to other seasonal conditions. One of the perceived risks of SDCT is that historically important pathogens like *Staphylococcus aureus* and *Streptococcus agalactiae* could gradually increase in prevalence over multiple lactations, which would not be identified in the current SDCT trial. Consequently, we recommend that herds implementing SDCT conduct routine surveillance for these pathogens using individual cow and bulk milk testing, in addition to monitoring DHIA SCC data for changes in infection dynamics.

3.5.7 Future Research

Further work in this multi-site project will investigate: 1) the effect of SDCT on cow-level indicators of health and productivity until 120 DIM; 2) test characteristics of the Minnesota Easy® 4Cast® plate and our algorithm; 3) the economic impact of SDCT; and

4) the microbiome impact of SDCT. Further research of SDCT as implemented by farm staff (and not research technicians) would further evaluate the suitability of SDCT on U.S. dairy farms. In addition, more work is needed to evaluate herd factors that impact the suitability of SDCT. Finally, more research is necessary to refine the existing SDCT protocols evaluated in this study, in order to further reduce antibiotic use. For example, research is needed to evaluate protocols that use narrow spectrum antibiotics, or that omit certain IMI from treatment such as NAS.

3.5.8 Conclusion

In a multi-site, randomized, natural exposure, non-inferiority trial, culture- and algorithm-guided SDCT each reduced antibiotic use by 55% without having a marked impact on risk of IMI cure and new IMI during the dry period and IMI prevalence at 1-13 DIM. The findings from this study and others indicate that SDCT can be successfully implemented on appropriate commercial dairy herds in the U.S.

3.6 Declaration of competing interest

The Minnesota Easy® 4Cast® plate is manufactured by the University of Minnesota (St. Paul, MN). However, the study investigators have no financial interest in the sale of this plate.

3.7 Authors' roles

SM. Rowe was involved in local and multi-site coordination, fieldwork, laboratory work, data management, statistical analysis and manuscript preparation. SM. Godden was

involved in study conceptualization, herd recruitment in MN and WI, supervision of fieldwork and manuscript editing. P. Gorden and A. Lago were involved in study conceptualization, local site coordination, fieldwork and manuscript editing. DV. Nydam was involved in study conceptualization, NY herd recruitment, local site coordination, and manuscript editing. AK. Vasquez was involved in fieldwork, local site coordination and manuscript editing. E. Royster was involved in study conceptualization and manuscript editing. J. Timmerman was involved in laboratory work and manuscript editing. MJ. Thomas was involved in local site coordination, fieldwork and manuscript editing.

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Thomas). We also thank Richard Maclehose and Darin Erickson for their input on statistical methods.

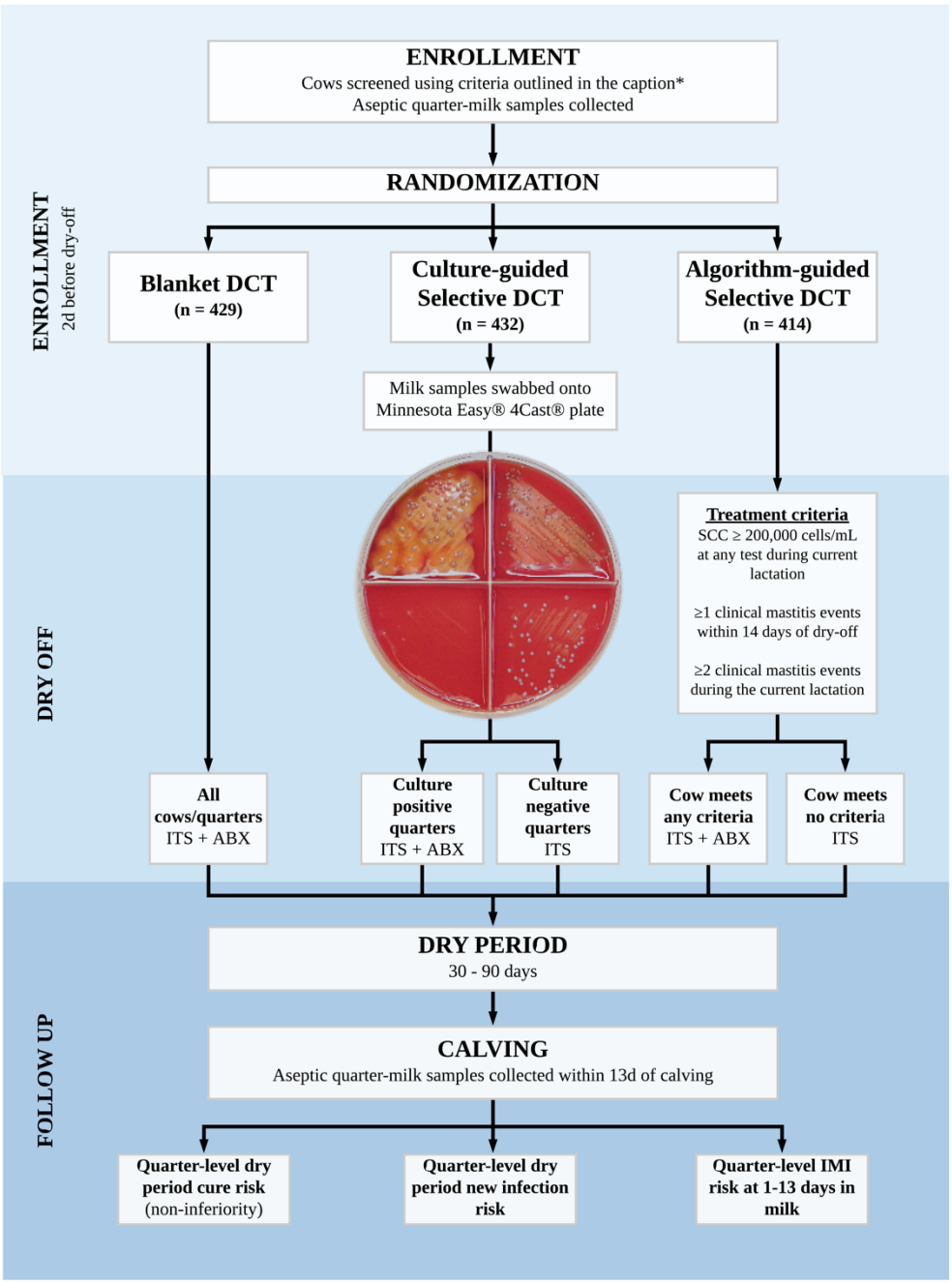

Figure 3.1: Outline of how treatments were allocated to cows in each treatment group, and the data collected to establish outcomes. ITS = Internal teat sealant (Orbeseal, Zoetis). ABX = Antibiotic treatment with 500 mg Ceftiofur hydrochloride (Spectramast DC, Zoetis). *To be enrolled, cows needed to have 4 functional quarters, an expected dry period length of 30 to 90 days, no recent antibiotic or anti-inflammatory treatment (within 14 d), no clinical mastitis, and not be lame or in poor body condition.

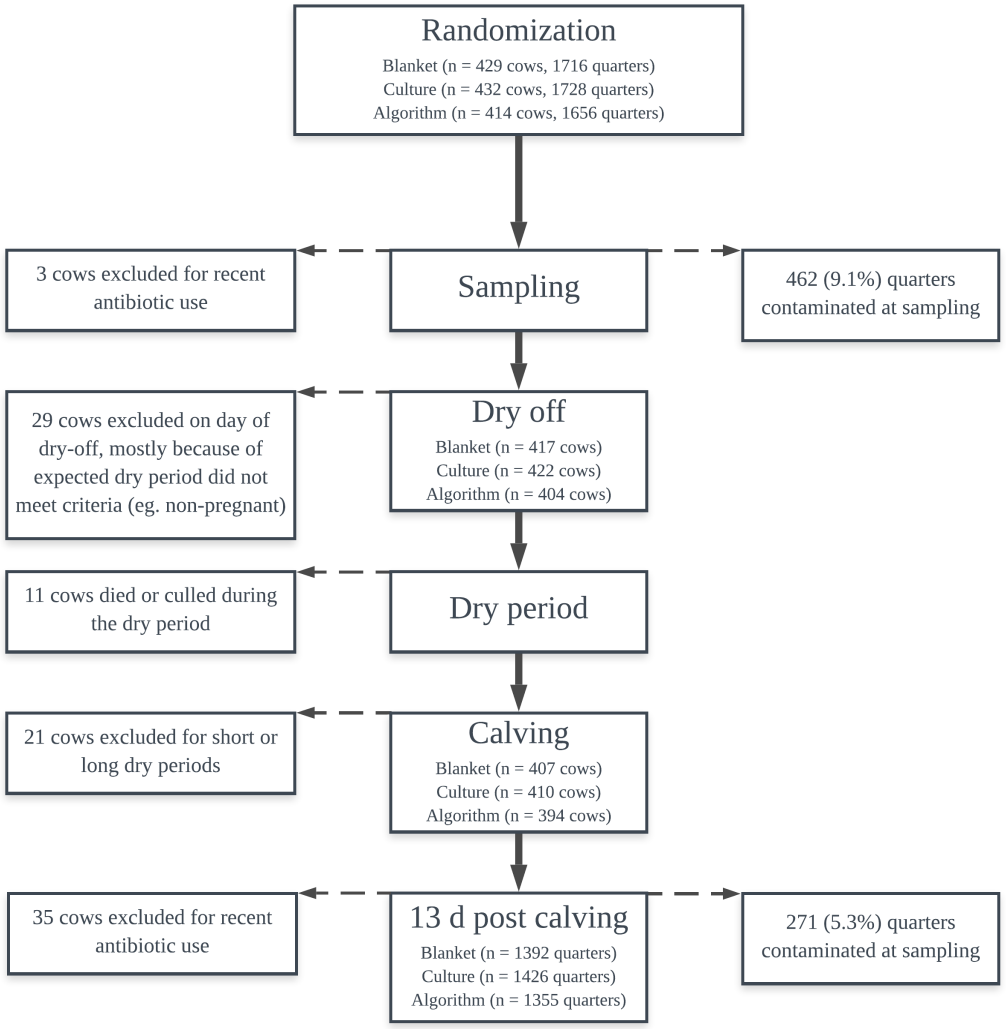

Figure 3.2: Description of losses to follow-up during each stage of the trial. Excluded cows (eg. for recent antibiotic use) are pictured to the left side of each phase of the study, while excluded quarters (mostly due to contamination) are pictured to the right side.

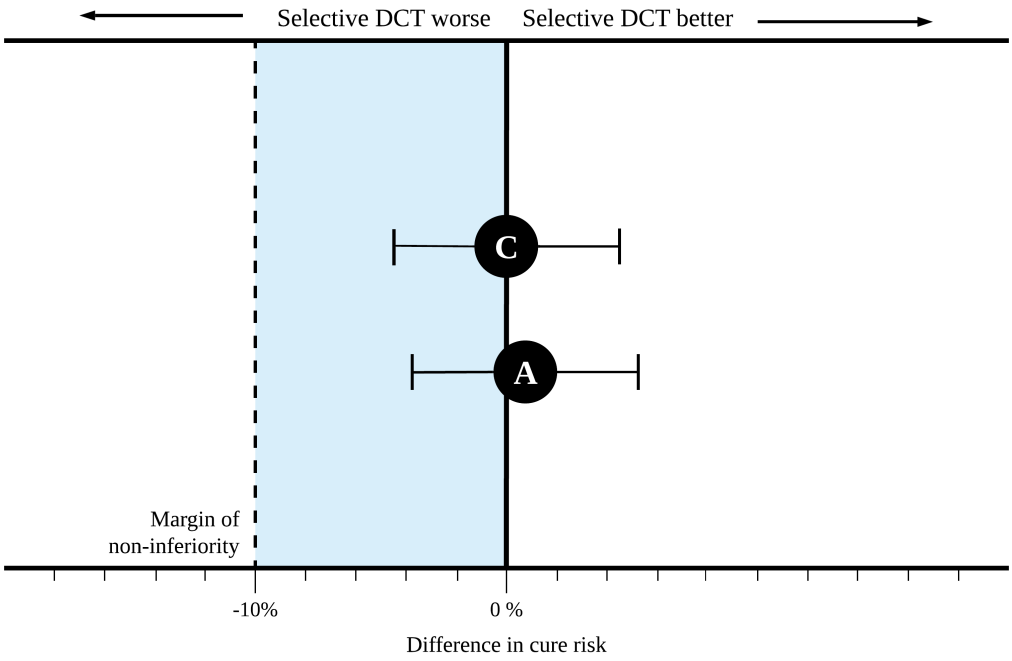

Figure 3.3: Non-inferiority analysis of dry period intramammary infection (IMI) cure risk for two selective dry cow therapy programs, as compared to blanket dry cow therapy. C = Culture. A = Algorithm. Adjusted dry period cure risk in the Blanket group was 89.7%, indicated by the solid vertical line. A margin or non-inferiority of -10% was specified *a priori*, which is indicated by the dashed vertical line. For Cure and Algorithm quarters, the lower limit of the two-sided 95% confidence intervals for the risk difference (cf. Blanket) was greater than the margin of non-inferiority, indicating that both selective dry cow therapy approaches are non-inferior to blanket dry cow therapy.

Table 3.1: Comparison of study herds for herd and cow-level characteristics at enrollment

	1 (n = 79)	2 (n = 86)	3 (n = 266)	4 (n = 393)	5 (n = 248)	6 (n = 54)	7 (n = 117)
Location	New York	New York	Iowa	California	California	Wisconsin	Minnesota
Predominant cow breed	Holstein	Holstein	Crossbred	Holstein	Holstein	Holstein	Holstein
Milking herd size	850	1150	1500	5700	3600	950	1750
Bulk tank SCC (x1,000 cells / ml)	200	100	150	230	220	110	90
Dry cow bedding system	Sand	Sand	Sand	MS	MS	Comp	Sand
Lactating cow bedding system	Sand	Sand	Sand / Comp	MS	MS	MS	Sand
Treatment group allocation							
Blanket	28 (35.4%)	29 (33.7%)	94 (35.3%)	125 (31.8%)	83 (33.4%)	17 (31.5%)	41 (35.0%)
Culture	28 (35.4%)	25 (29.1%)	88 (33.1%)	137 (34.9%)	86 (34.7%)	19 (35.2%)	39 (33.3%)
Algorithm	23 (29.2%)	32 (37.2%)	84 (31.6%)	131 (33.3%)	79 (31.9%)	18 (33.3%)	37 (31.6%)
Median [IQR] Age at enrollment (mo)	44 [33, 60]	45 [35, 57]	45 [34, 56]	45 [34, 57]	45 [34, 56]	44 [33, 48]	45 [33, 57]
Mean (SD) milk yield at last test (kg/d)[1]	23.0 (6.23)	26.2 (9.74)	29.5 (6.89)	30.4 (7.83)	19.8 (8.51)	30.2 (5.04)	30.3 (6.45)
Mean (SD) days in milk at dry-off	347 (51.8)	336 (53.1)	314 (37.8)	325 (44.3)	329 (50.7)	328 (42.5)	321 (38.5)
Mean (SD) SCC ($\log_e$ cells x 10^3)[2]	4.48 (1.06)	4.09 (1.13)	4.00 (0.906)	4.46 (1.16)	5.17 (1.38)	4.36 (0.934)	3.95 (1.09)
Mean (SD) peak $\log_e$ SCC (log cells x 10^3)[3]	5.50 (1.34)	5.12 (1.35)	4.77 (1.08)	5.76 (1.20)	6.12 (1.10)	5.03 (0.898)	4.76 (1.16)
Clinical mastitis during lactation of enrollment							
None	64 (81.0%)	84 (97.7%)	230 (86.5%)	340 (86.5%)	196 (79.0%)	53 (98.1%)	104 (88.9%)
≥1	15 (19.0%)	2 (2.3%)	36 (13.5%)	53 (13.5%)	52 (21.0%)	1 (1.9%)	13 (11.1%)
Parity at enrollment							
1	32 (40.5%)	34 (39.5%)	94 (35.3%)	183 (46.5%)	105 (42.3%)	25 (46.3%)	47 (40.2%)
2	16 (20.3%)	28 (32.6%)	96 (36.1%)	102 (26.0%)	75 (30.2%)	20 (37.0%)	36 (30.8%)
≥3	31 (39.2%)	24 (27.9%)	76 (28.6%)	108 (27.5%)	68 (27.5%)	9 (16.7%)	34 (29.1%)

[1]Milk yield at the most recent herd test before enrollment, [2]Somatic cell count at the most recent herd test before enrollment, [3]Highest log somatic cell count at any point during the lactation of enrollment

Comp = Compost Pack. MS = Manure solids.

Table 3.2: Comparison of treatment groups for cow-level characteristics at enrollment

	Blanket (n=417)	Culture (n=422)	Algorithm (n=404)	Overall (n=1243)
Median [IQR] Age at enrollment (months)	45 [34, 57]	44 [34, 57]	45 [34, 56]	45 [34, 56]
Mean (SD) milk yield at last test (kg/d)[1]	26.8 (8.87)	27.8 (8.81)	27.3 (8.38)	27.3 (8.70)
Mean (SD) dry period length (days)	55.4 (7.92)	55.9 (7.61)	55.9 (8.16)	55.7 (7.89)
Mean (SD) SCC at last test (log_e cells x 1,000 per ml)[2]	4.45 (1.21)	4.39 (1.22)	4.45 (1.20)	4.43 (1.21)
Mean (SD) peak log SCC (log_e cells x 1,000 per ml)[3]	5.39 (1.31)	5.51 (1.23)	5.40 (1.28)	5.43 (1.27)
Clinical mastitis during lactation of enrollment				
None	358 (85.9%)	359 (85.1%)	354 (87.6%)	1071 (86.2%)
≥1	59 (14.1%)	63 (14.9%)	50 (12.4%)	172 (13.8%)
Parity at enrollment				
1	163 (39.1%)	188 (44.5%)	169 (41.8%)	520 (41.8%)
2	135 (32.4%)	111 (26.3%)	127 (31.4%)	373 (30.0%)
≥3	119 (28.5%)	123 (29.2%)	108 (26.8%)	350 (28.2%)

[1]Milk yield at the most recent herd test before enrollment
[2]Somatic cell count at the most recent herd test before enrollment
[3]Highest log_e somatic cell count at any point during the lactation of enrollment

Table 3.3: Quarter-level prevalence of intramammary infection from 1275 enrolled cows, two days before planned dry-off date, stratified by treatment group

	Blanket		Culture		Algorithm		All quarters	
	n	%	n	%	n	%	n	%
Total quarters enrolled	1716		1728		1656		5100	
Quarters excluded[1]	148	8.62	136	7.87	112	6.76	396	7.76
Contaminated	172	10.02	150	8.68	140	8.45	462	9.06
Quarters at risk	1396	100.00	1442	100.00	1404	100.00	4242	100.00
No Growth	1046	74.93	1069	72.54	1049	74.50	3164	74.59
Infected quarters	350	25.07	373	25.87	355	25.28	1078	25.41
Single pathogen	322	23.07	340	23.58	325	23.15	987	23.27
Mixed infection	28	2.01	33	2.29	30	2.14	91	2.15
Gram Positive	334	23.93	360	24.97	330	23.50	1024	24.14
Staph. aureus	2	0.14	3	0.21	6	0.43	11	0.26
Non aureus Staph spp.	198	14.18	228	15.81	203	14.46	629	14.83
Staph. chromogenes	110	7.88	124	8.60	106	7.55	340	8.02
Staph. epidermidis	10	0.72	5	0.35	8	0.57	23	0.54
Staph. gallinarum	0	0.00	1	0.07	0	0.00	1	0.02
Staph. haemolyticus	12	0.86	18	1.25	17	1.21	47	1.11
Staph. hominis	2	0.14	0	0.00	0	0.00	2	0.05
Staph. sciuri	4	0.29	7	0.49	7	0.50	18	0.42
Staph. simulans	1	0.07	3	0.21	2	0.14	6	0.14
Staph. xylosus	15	1.07	12	0.83	12	0.85	39	0.92
Staph. sp.	52	3.72	63	4.37	57	4.06	172	4.05
Strep & Strep-like	33	2.36	30	2.08	28	1.99	91	2.15
Aerococcus viridans	0	0.00	2	0.14	2	0.14	4	0.09
Aerococcus sp.	1	0.07	2	0.14	2	0.14	5	0.12
Enterococcus faecalis	1	0.07	0	0.00	1	0.07	2	0.05
Enterococcus hirae	1	0.07	1	0.07	0	0.00	2	0.05
Enterococcus sp.	2	0.14	3	0.21	2	0.14	7	0.17
Lactococcus garvieae	5	0.36	2	0.14	1	0.07	8	0.19
Lactococcus lactis	2	0.14	0	0.00	1	0.07	3	0.07
Lactococcus sp.	4	0.29	3	0.21	5	0.36	12	0.28
Streptococcus mitis	3	0.21	2	0.14	1	0.07	6	0.14
Streptococcus oralis	0	0.00	0	0.00	2	0.14	2	0.05
Streptococcus uberis	1	0.07	2	0.14	2	0.14	5	0.12
Streptococcus sp.	13	0.93	15	1.04	10	0.71	38	0.90
Other Gram Positive	114	8.17	115	7.98	104	7.41	333	7.85
Actinomyces sp.	0	0.00	2	0.14	1	0.07	3	0.07
Bacillus sp.	17	1.22	14	0.97	8	0.57	39	0.92
Corynebacterium sp.	56	4.01	61	4.23	53	3.77	170	4.01
Gram positive coccus	2	0.14	3	0.21	6	0.43	11	0.26
Gram positive organism	5	0.36	3	0.21	3	0.21	11	0.26
Gram positive rod	6	0.43	10	0.69	8	0.57	24	0.57
Kocuria sp.	0	0.00	2	0.14	2	0.14	4	0.09
Lactobacillus sp.	0	0.00	1	0.07	2	0.14	3	0.07
Micrococcus luteus	1	0.07	1	0.07	3	0.21	5	0.12

Micrococcus sp.	25	1.79	21	1.46	16	1.14	62	1.46
Paenibacillus sp.	1	0.07	2	0.14	1	0.07	4	0.09
Rothia sp.	2	0.14	2	0.14	4	0.28	8	0.19
Streptomyces sp.	0	0.00	0	0.00	2	0.14	2	0.05
Trueperella pyogenes	1	0.07	0	0.00	1	0.07	2	0.05
Gram Negative	18	1.29	15	1.04	30	2.14	63	1.49
Coliform	11	0.79	4	0.28	10	0.71	25	0.59
Enterobacter aerogenes	1	0.07	0	0.00	0	0.00	1	0.02
Enterobacter cloacae	5	0.36	1	0.07	4	0.28	10	0.24
Enterobacter kobei	1	0.07	0	0.00	0	0.00	1	0.02
Escherichia coli	1	0.07	2	0.14	4	0.28	7	0.17
Escherichia sp.	0	0.00	0	0.00	1	0.07	1	0.02
Klebsiella oxytoca	1	0.07	0	0.00	0	0.00	1	0.02
Klebsiella pneumoniae	2	0.14	1	0.07	1	0.07	4	0.09
Other Gram Negative	7	0.50	11	0.76	20	1.42	38	0.90
Other Yeast	2	0.14	1	0.07	0	0.00	3	0.07

[1]Quarters from cows that were excluded during the trial or retrospectively. Most exclusions were due to failure of subjects to calve 30 to 90 days after planned dry-off and antibiotic treatment less than 15 days prior to sampling.

Table 3.4: Quarter-level prevalence of intramammary infection from 1275 enrolled cows at 1-13 days after calving, stratified by treatment group

	Blanket		Culture		Algorithm		All quarters	
	n		n	%	n	%	n	%
Total quarters enrolled	1716		1728		1656		5100	
Quarters excluded from analysis[1]	224	13.05%	212	12.27%	220	13.35%	396	7.76%
Contaminated at 1-13 DIM	100	5.83%	90	5.21%	81	4.83%	271	5.31%
Quarters at risk	1392	100.00%	1426	100.00%	1355	100.00%	4173	100.00%
No Growth	1075	77.19%	1085	75.39%	1052	79.54%	3212	79.54%
Infected quarters	317	22.77%	341	23.91%	303	22.36%	961	23.03%
Single pathogen	272	19.54%	311	21.81%	271	20.00%	854	20.46%
Mixed infection	45	3.23%	30	2.10%	32	2.36%	107	2.56%
Gram Positive	289	20.76%	311	21.81%	286	21.11%	886	21.23%
Staph. aureus	2	0.14%	0	0.00%	0	0.00%	2	0.05%
Non-aureus *Staph. spp.*	174	12.50%	196	13.74%	174	12.84%	544	13.04%
Staph. chromogenes	63	4.53%	71	4.98%	58	4.28%	192	4.60%
Staph. epidermidis	0	0.00%	0	0.00%	2	0.15%	2	0.05%
Staph. gallinarum	1	0.07%	0	0.00%	0	0.00%	1	0.02%
Staph. haemolyticus	13	0.93%	12	0.84%	8	0.59%	33	0.79%
Staph. hominis	0	0.00%	1	0.07%	1	0.07%	2	0.05%
Staph. sciuri	44	3.16%	43	3.02%	41	3.03%	128	3.07%
Staph. simulans	0	0.00%	2	0.14%	2	0.15%	4	0.10%
Staph. xylosus	1	0.07%	6	0.42%	6	0.44%	13	0.31%
Staph. sp.	63	4.53%	68	4.77%	64	4.72%	195	4.67%
Strep and Strep-like	17	1.22%	17	1.19%	23	1.70%	57	1.37%
Aerococcus viridans	2	0.14%	1	0.07%	2	0.15%	5	0.12%
Aerococcus sp.	1	0.07%	2	0.14%	0	0.00%	3	0.07%
Enterococcus faecalis	1	0.07%	1	0.07%	0	0.00%	2	0.05%
E. saccharolyticus	1	0.07%	0	0.00%	1	0.07%	2	0.05%
E. thailandicus	0	0.00%	1	0.07%	0	0.00%	1	0.02%
Enterococcus sp.	1	0.07%	1	0.07%	1	0.07%	3	0.07%
Lactococcus garvieae	2	0.14%	2	0.14%	2	0.15%	6	0.14%
Lactococcus lactis	0	0.00%	1	0.07%	0	0.00%	1	0.02%
Lactococcus sp.	2	0.14%	0	0.00%	4	0.30%	6	0.14%
Strep. dysgalactiae	0	0.00%	2	0.14%	2	0.15%	4	0.10%
Strep. gallinarum	1	0.07%	0	0.00%	0	0.00%	1	0.02%
Strep. mitis	2	0.14%	3	0.21%	2	0.15%	7	0.17%
Strep. uberis	1	0.07%	1	0.07%	1	0.07%	3	0.07%
Strep. sp.	4	0.29%	4	0.28%	9	0.66%	17	0.41%
Other Gram Positive	115	8.26%	112	7.85%	103	7.60%	330	7.91%
Arthrobacter sp.	1	0.07%	0	0.00%	1	0.07%	2	0.05%
Bacillus sp.	48	3.45%	47	3.30%	36	2.66%	131	3.14%

Corynebacterium sp.	38	2.73%	34	2.38%	30	2.21%	102	2.44%
Gram positive coccus	6	0.43%	4	0.28%	8	0.59%	18	0.43%
Gram positive organism	2	0.14%	1	0.07%	3	0.22%	6	0.14%
Gram positive rod	12	0.86%	18	1.26%	14	1.03%	44	1.05%
Lactobacillus sp.	0	0.00%	1	0.07%	0	0.00%	1	0.02%
Micrococcus luteus	1	0.07%	1	0.07%	3	0.22%	5	0.12%
Micrococcus sp.	1	0.07%	2	0.14%	5	0.37%	8	0.19%
Paenibacillus sp.	0	0.00%	1	0.07%	1	0.07%	2	0.05%
Rothia sp.	9	0.65%	6	0.42%	5	0.37%	20	0.48%
Trueperella pyogenes	0	0.00%	0	0.00%	1	0.07%	1	0.02%
Gram Negative	33	2.37%	28	1.96%	18	1.33%	79	1.89%
Coliform	6	0.43%	10	0.70%	2	0.15%	18	0.43%
Enterobacter cloacae	2	0.14%	2	0.14%	1	0.07%	5	0.12%
Escherichia coli	3	0.22%	5	0.35%	1	0.07%	9	0.22%
Klebsiella oxytoca	1	0.07%	2	0.14%	0	0.00%	3	0.07%
Klebsiella pneumoniae	0	0.00%	1	0.07%	0	0.00%	1	0.02%
Other Gram Negative	27	1.94%	18	1.26%	16	1.18%	61	1.46%
Other								
Prototheca sp.	3	0.22%	4	0.28%	1	0.07%	8	0.19%
Yeast	1	0.07%	2	0.14%	3	0.22%	6	0.14%

[1]Quarters from cows that were excluded during the trial or retrospectively. Most exclusions were due to failure of subjects to calve 30 to 90 days after planned dry-off, antibiotic treatment less than 15 days prior to sampling and failure to collect a sample within 13 days of calving.

Table 3.5: Unconditional associations for possible predictors of quarter-level measures of udder health during the dry period.

	IMI at enrollment (n at risk = 4242)		IMI at 1 – 13d DIM (n at risk = 4173)		Dry period new IMI (n at risk = 3794)		Dry period IMI cure (n at risk = 935)	
	Risk	**Risk Ratio (95% CI)**	**Risk**	**Risk Ratio (95% CI)**	**Risk**	**Risk Ratio (95% CI)**	**Risk**	**Risk Ratio (95% CI)**
All quarters	25.4%		23.0%		20.1%		87.5%	
Treatment group								
Blanket	25.1%	Ref	22.8%	Ref	19.6%	Ref	86.8%	Ref
Culture	25.9%	1.04 (0.88, 1.23)	23.9%	1.07 (0.90, 1.27)	21.0%	1.09 (0.90, 1.32)	87.5%	1.01 (0.95, 1.07)
Algorithm	25.3%	1.01 (0.85, 1.20)	22.4%	0.98 (0.82, 1.17)	19.8%	1.01 (0.83, 1.24)	88.1%	1.02 (0.96, 1.08)
Age (years)		1.01 (0.96, 1.05)		1.08 (1.03, 1.13)		1.09 (1.04, 1.15)		1.01 (0.99, 1.03)
Parity at enrollment								
1	23.7%	Ref	21.4%	Ref	18.7%	Ref	88.4%	Ref
2	28.8%	1.30 (1.11, 1.53)	23.8%	1.15 (0.97, 1.36)	20.5%	1.12 (0.93, 1.36)	85.7%	0.97 (0.91, 1.03)
≥3	24.4%	1.04 (0.88, 1.24)	25.0%	1.22 (1.02, 1.46)	22.3%	1.25 (1.03, 1.51)	88.3%	1.00 (0.94, 1.06)
DIM at dry-off (per 10d)[1]		0.98 (0.96, 0.99)		1.01 (0.99, 1.02)		1.01 (0.99, 1.03)		1.00 (0.99, 1.00)
Milk yield at last test (kg)		1.03 (1.02, 1.04)		1.00 (0.99, 1.01)		1.00 (0.99, 1.01)		1.00 (1.00, 1.00)
SCC at last test ($\log_e$ cells x 1,000 per ml)		1.09 (1.03, 1.15)		1.14 (1.08, 1.21)		1.15 (1.08, 1.22)		0.99 (0.97, 1.01)
Peak SCC during lactation of enrollment ($\log_e$ cells x 1,000 per ml)		1.07 (1.03, 1.12)		1.12 (1.07, 1.16)		1.12 (1.07, 1.17)		0.98 (0.96, 1.00)
SCC at last test								

<200,000	24.9%	Ref	21.9%	Ref	18.8%	Ref	87.3%	Ref
≥200,000	27.1%	1.12 (0.95, 1.32)	27.1%	1.33 (1.12, 1.57)	24.4%	1.39 (1.16, 1.66)	88.2%	1.01 (0.96, 1.07)
Clinical mastitis during lactation of enrollment								
No cases	24.6%	Ref	22.5%	Ref	19.4%	Ref	86.9%	Ref
≥1 cases	30.8%	1.37 (1.13, 1.65)	26.4%	1.24 (1.01, 1.51)	24.7%	1.36 (1.09, 1.68)	90.3%	1.04 (0.98, 1.10)
Dry period length (per 10 days)		1.06 (0.99, 1.13)		1.05 (0.99, 1.14)		1.13 (1.00, 1.27)		1.01 (0.98, 1.05)

[1]Risk ratio estimates are per every 10 days. For example RR for cure = 0.98, means that risk of risk of intramammary infection at enrollment is 2% less for every additional 10 days in milk at dry-off.

Table 3.6: Final generalized linear mixed models (logistic) estimating the effect of two selective dry cow therapy programs (compared with blanket dry cow therapy) on dry period intramammary infection dynamics.

	Adjusted Risk[1]	β	SE	Risk Difference[2]	95% CI of Risk Difference
Dry period IMI[3] cure					
Blanket	89.7%	Ref			
Culture	89.9%	0.027	0.293	+0.2%	-4.4, 4.8%
Algorithm	90.6%	0.114	0.302	+0.9%	-3.7, 5.5%
Dry period new IMI					
Blanket	14.7%	Ref			
Culture	15.2%	0.046	0.124	+0.5%	-2.2, 3.2%
Algorithm	14.7%	0.004	0.127	+0.0%	-2.6, 2.7%
IMI post calving					
Blanket	17.2%	Ref			
Culture	17.5%	0.028	0.119	+0.3%	-2.4, 3.1%
Algorithm	16.7%	-0.038	0.122	-0.5%	-3.2, 2.3%

[1]Risk estimates using predicted/estimated marginal means (~ least squares means)
[2]Risk difference estimates and 95% confidence intervals were derived from generalized linear mixed models using predicted/estimated marginal means in STATA (aka "marginal standardization")
[3]IMI = Intramammary infection
Final model included random intercepts for cow and herd. All fixed-effect covariates were removed from the final model, as there was no evidence for confounding when using the 10% change in estimate approach.
Intraclass correlation coefficient for Dry period IMI cure for cow and herd were 0.31 and 0.04 respectively.
Intraclass correlation coefficient for Dry period new IMI for cow and herd were 0.28 and 0.15 respectively.
Intraclass correlation coefficient for IMI at calving for cow and herd were 0.31 and 0.16 respectively.

4 CHAPTER FOUR: Randomized Controlled Trial Investigating the Effect of Two Selective Dry Cow Therapy Protocols on Udder Health and Performance in the Subsequent Lactation

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4.1 Summary

The objective of this study was to compare culture- and algorithm-guided selective dry cow therapy (**SDCT**) programs to blanket dry cow therapy (**BDCT**) in a multi-site, randomized, natural exposure, clinical trial for the following cow-level outcomes: clinical mastitis, removal from the herd, and DHIA test-day milk yield and SCC measures during the first 120 days in milk (**DIM**). Two days before planned dry-off, cows in each of 7 herds were randomly allocated to BDCT ("Blanket"), culture-guided SDCT ("Culture") or algorithm-guided SDCT ("Algorithm"). At dry-off, Blanket cows received an intramammary antibiotic (500mg ceftiofur hydrochloride) in all four quarters. Antibiotic treatments were selectively allocated to quarters of Culture cows by only treating quarters from which aseptically collected milk samples tested positive on the a rapid culture system after 30-40 hours of incubation. For Algorithm cows, antibiotic treatments were selectively allocated at the cow-level, with all quarters receiving antibiotic treatment if the cow met at least one of the following criteria: 1) any Dairy Herd Improvement Association (**DHIA**) test with a somatic cell count (**SCC**) > 200,000 cells / ml during the current lactation; 2) two or more clinical mastitis cases during the current lactation; and 3) one or more clinical mastitis cases in the 14 days before enrollment. All quarters of all cows were treated with an internal teat sealant (**ITS**). Clinical mastitis and removal from

the herd events (i.e. culling or death), and DHIA test-day data from dry-off to 120 DIM were extracted from herd records. Hazard ratios (**HR**) for the effect of treatment group on clinical mastitis and removal from the herd during 1-120 DIM were determined using Cox proportional hazards regression. The effects of treatment group on test-day $\log_e$-transformed SCC and milk yield were determined using linear mixed models. Final models indicated that either SDCT program is unlikely to increase clinical mastitis risk ($HR_{Culture/Blanket}$ = 0.82, 95% CI: 0.58, 1.15 and $HR_{Algorithm/Blanket}$ = 0.83, 95% CI: 0.63, 1.09) or test-day $\log_e$SCC (Culture minus Blanket = 0.05 95% CI: -0.09, 0.18 and Algorithm minus Blanket = 0.07 95% CI: -0.07, 0.21). Removal from the herd risk and test-day milk yield were also similar between treatment groups. Findings from this study indicate that culture- or algorithm-guided SDCT can be used at dry-off without negatively impacting cow health and performance in early lactation.

4.2 Introduction

Udder health in early lactation can be improved by treating cows with long-acting antibiotics at the time of dry-off (dry cow therapy, **DCT**). In particular, DCT has been shown to reduce clinical mastitis risk and SCC in early lactation (Macmillan et al., 1983, Schukken et al., 1993, Hogan et al., 1994, Bradley and Green, 2001), both of which are associated with reduced milk production and increased rate of removal from the herd (Jones et al., 1984, Seegers et al., 2003, De Vliegher et al., 2005, Gröhn et al., 2005). However, BDCT contributes to almost one third of total antibiotic use in lactating cows on conventional dairy farms in the United States (Pol and Ruegg, 2007), and given that consumers have increasing concerns about antibiotic stewardship in food animal

production, there is great interest to increase the adoption of antibiotic-sparing approaches to DCT. Despite the potential of SDCT to improve antibiotic stewardship in the U.S. dairy industry, only 10% of herds practice SDCT (USDA-NAHMS, 2014), which may, in part, be due to perceived risks to cow health. Such concerns may be in response to the failure of early SDCT approaches, which increased clinical mastitis risk and SCC in early lactation (Berry and Hillerton, 2002, McDougall, 2010, Rajala-Schultz et al., 2011, Scherpenzeel et al., 2014). However, more recent trials that evaluated SDCT programs which included the use of teat sealants found negligible effects on these measures of udder health shortly after calving (Bradley et al., 2010, Cameron et al., 2015, Vasquez et al., 2018). However, many of the aforementioned studies were conducted at a single site, and the one favorable study conducted in the U.S. enrolled cows from a single herd, thus limiting the generalizability of their findings. Another limitation of some of the aforementioned SDCT studies was failure to measure the impact of the SDCT program on longer-term indicators of cow-level health and performance that are clinically and economically relevant to producers. For example, Vasquez et al. (2018) reported on udder health for the first month post-calving. Bradley et al. (2010) reported quarter infection status for 100 days post-calving but, because the study used a split udder design, was not able to report on overall cow health and performance outcomes measured at the cow-level. Finally, no studies to date have made direct comparisons to culture and algorithm-guided SDCT programs in commercial dairy herds. Consequently, there is a need for a multi-site field trial that compares BDCT to different SDCT programs. In an earlier companion manuscript, we reported on a multi-site randomized clinical trial investigating the effects of using a culture and algorithm-guided SDCT programs (vs

BDCT) on antibiotic use and quarter-level infection dynamics during the dry period (Rowe et al., Submitted-a). We found that the two SDCT approaches (culture- and algorithm-guided) each reduced antibiotic use at dry-off by 55%, without causing any negative impacts on intramammary infection (**IMI**) dynamics during the dry period. The objective of the current manuscript was to compare the culture and algorithm-guided SDCT programs to BDCT for longer term cow-level outcomes, including clinical mastitis risk, removal from the herd risk, and test-day milk yield and SCC during the first 120 days of lactation. We hypothesized that implementing either of the two SDCT programs would have no negative impact on clinically relevant outcomes including clinical mastitis risk, removal from the herd risk, and DHIA SCC and milk production measures during the first 120 days of lactation.

4.3 Materials and Methods

A randomized, controlled, natural exposure study of SDCT in U.S. dairy herds was conducted between May 2018 and April 2019. The REFLECT guidelines were followed in the reporting of this study (O'Connor et al., 2010). Ethics approval was granted by the University of Minnesota Institutional Animal Care and Use Committee (#1801-35489A).

4.3.1 Study Herds

Seven herds were recruited from five states (California = 2, Iowa = 1, Minnesota = 1, New York = 2, Wisconsin = 1), from May to July, 2018. Herds were selected because they had a working relationship with University of Minnesota, Cornell University, Iowa State University, DairyExperts, Inc. or Dairy Health & Management Services, had a herd

size sufficiently large to dry off at least 15 cows per week, had an average bulk milk SCC less than 250,000 cells per ml during the previous 12 months, were on a monthly DHIA testing schedule (i.e. individual cow SCC and milk weight measurements), and consistently and accurately recorded clinical mastitis, culling and death events. The bulk tank SCC threshold was used as an inclusion criteria as it is suggested by mastitis experts that lower bulk milk SCC herds may be more appropriate for the implementation of SDCT than high SCC herds (Bradley et al., 2018).

4.3.2 Cow enrollment visit

Study technicians visited herds each week to enroll cows two days prior to the planned dry-off date. All cows were enrolled between May and August 2018. Cows were eligible for enrollment if they had 4 functional quarters, an expected dry period length of 30 to 90 days, no recent antibiotic or anti-inflammatory treatment (within 14 d), no clinical mastitis (i.e. no visible abnormalities present in foremilk or heat, erythema or pain detected upon palpation of the udder) and were not lame (< 4/5; Sprecher et al. (1997)) or in poor body condition (≥ 2/5; Edmonson et al. (1989)). Following enrollment, duplicate, aseptic quarter-milk samples were collected from enrolled cows according to National Mastitis Council guidelines (NMC, 2017). Briefly, after farm staff performed their usual pre-milking teat disinfection routine, study technicians, who were wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs, discarded three squirts of foremilk and sampled approximately 20-30 ml of milk into sterile 60-ml vials. Samples were immediately chilled on ice and transported back to the site laboratory.

Cows were block-randomized (block size = 18, number of unique blocks = 11) to one of three treatment groups by study technicians: Blanket DCT ("Blanket"), Culture-based SDCT ("Culture"), and Algorithm-based SDCT ("Algorithm"). Block randomization ensures equal allocation to groups at a given enrollment time or place, which in this study, allowed us to stratify our randomization by herd and dry-off date. The sequence of treatment groups within each block was determined using a random number generator in a commercial spreadsheet program (Microsoft Excel, Redmond, WA), which was printed onto the set of enrollment forms used at all sites. Randomization was stratified by herd and enrollment date. Following enrollment, cows were milked by farm staff according to each farm's usual routine until the day of dry-off, two days later. On two farms (Herds 5 and 6), cows were milked once daily until dry-off. For Herd 6, a commercial external teat sealant product (T-Hexx Dry, Huvepharma) was applied to the teats after each milking. Other herds were milked two or three-times daily until dry-off.

4.3.3 Rapid culture

Upon arrival back to the site laboratory, quarter milk samples from cows in the Culture group were plated onto a rapid culture system (Minnesota Easy® 4Cast® plate, St Paul, Minnesota) using sterile cotton-tip swabs and incubated at 37 ± 2°C for 30-40hrs. All milk samples, including those used for inoculating the rapid culture plates, were stored at -20°C for later laboratory analysis. Rapid culture plates were read by a study technician the morning of the dry-off visit, and quarter-level results recorded as 'growth' or 'no growth'. Samples with growth patterns suggestive of contamination (i.e. numerous independent isolates) were classified as growth. We chose to treat all IMI, including 'major' vs 'minor' pathogens in order to make the system as simple as possible to

facilitate on-farm implementation. Furthermore, it is currently unknown which IMI are likely to benefit or not benefit from antibiotic treatment at dry-off.

4.3.4 Dry-off visit

Dry-off was conducted two days after enrollment. All quarters of Blanket cows were treated with an intramammary antibiotic (500mg ceftiofur hydrochloride, SPECTRAMAST® DC, Zoetis. Parsippany, NJ). In Culture cows, individual quarters were treated with antibiotics if any growth was observed on the rapid culture plate. Algorithm cows had all 4 quarters treated with antibiotics if they met any of following criteria for treatment: ≥2 cases of clinical mastitis during lactation, 1 case of clinical mastitis during the 14 day period preceding dry-off, or any DHIA test with a SCC > 200,000 cells/ml during lactation. We chose a threshold of ≥2 cases for clinical mastitis based on the hypothesis that a higher threshold (i.e. 2 instead of 1) would improve the specificity of the algorithm and thus reduce unnecessary antibiotic use at dry-off. All quarters of all cows were infused with an ITS containing bismuth subnitrate (ORBESEAL®, Zoetis. Parsippany, NJ). Antibiotic and ITS infusions were administered after the final milking in the following fashion: study technicians, who were wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs for at least 5 seconds before the antibiotic treatment was infused into the mammary gland and again before ITS was infused into the teat cistern. A partial insertion technique was used for both antibiotic and ITS treatments. Cows that received the incorrect antibiotic treatments were either excluded or reassigned to the BDCT group.

4.3.5 Follow-up during the Dry Period and Post-Calving

Quarter-level IMI dynamics were determined through collecting and culturing aseptic quarter milk samples between 1-13 DIM (Rowe et al., Submitted-b). Clinical diseases occurring during the dry period and early lactation were detected and recorded by farm staff who were masked to treatment status of cows and laboratory culture results, with the exception of two farms where the herd manager was granted access to antibiotic treatment records, which could have allowed them to indirectly determine treatment status of cows. Somatic cell count and milk yield data were measured at monthly intervals as part of the herd's regular DHIA schedule, and along with health event data, were extracted from the electronic herd records.

4.3.6 Statistical Analysis

4.3.6.1 Sample Size Calculation

Sample size was calculated to enable non-inferiority analysis of quarter-level IMI cure risk during the dry period as previously reported in Rowe et al. (Submitted-b).

4.3.6.2 Variable management

Cow- and quarter-level enrollment and dry-off treatment records, herd demographic information, and laboratory findings were recorded in spreadsheets (Google Sheets; Mountain View, CA). Disease events (clinical mastitis, culling, and death), test-day SCC, and milk yield data were extracted from electronic farm records. Data were imported into the R Statistical Programming Environment (R Core Team, 2018) for merging, cleaning, and analysis. An analysis log can be found at (https://samrowe101.github.io/SDCT-2019/Cowoutcomes.html). Normality of continuous variables was assessed by visualizing

normal quantile-quantile plots. All SCC measures were $\log_e$ transformed for analysis. Cows and quarters were retrospectively excluded from analysis if they failed to meet inclusion criteria. Consequently, cows with a dry period outside of the 30 to 90 day range, including cows that failed to calve or were culled during the dry period were excluded from analysis.

4.3.6.3 Effect of Selective Dry Cow Therapy on Risk for Clinical Mastitis and Risk for Removal from the Herd in the First 120 Days in Milk

Time to event analysis was conducted to determine the effect of SDCT on clinical mastitis and removal from the herd during the first 120 days of lactation. Clinical mastitis cases were defined as the first case of clinical mastitis from 1-120 DIM. Cows were left-censored (excluded) if they had died or been culled between enrollment and calving. Cows with clinical mastitis events during the dry period were considered at risk for clinical mastitis during 1-120 DIM. Cows were right-censored at 120 DIM or at removal from the herd. Cows were classified as having been removed from the herd if they died or were culled. Decisions to cull the animal in the future (eg. “Do not breed” or “DNB”) were not considered as culling events. Potential confounders were measured at the cow-level, including: age at enrollment, parity at enrollment (1, 2, ≥3), milk yield at the most recent herd test prior to enrollment (kg), $\log_e$ SCC at the most recent herd test prior to enrollment ($\log_e$[cells/1000] per ml), peak $\log_e$ SCC during lactation of enrollment, and clinical mastitis during the lactation of enrollment (dichotomous). Kaplan-Meier survival curves were generated, with the log-rank test used to compare between treatment groups. Hazard ratios were estimated using Cox proportional hazards regression, with a robust sandwich estimator used to account for the clustering of cows within herds. The

proportional hazards assumption for each covariate was assessed by visualizing Schoenfeld residuals against time and using the Schoenfeld test. Covariates with hazards shown to vary over time (beta $\neq 0$, $P < 0.05$) were stratified to allow for multiple baseline hazards. The Efron approximation method was used to deal with tied observations. Unconditional risk ratio (**RR**) estimates for the relationship between explanatory variables of interest and 120-day clinical mastitis risk or risk of removal from the herd were calculated using generalized linear models (binomial family, log link; aka 'log-binomial model').

A directed acyclic graph (DAG) was drawn for each model to identify potential confounders for inclusion as covariates into the multivariable models. Correlations between potential confounders were determined using Pearson's correlation coefficient and Kendall's Tau for normally and non-normally distributed continuous variables respectively. Highly correlated variables (> 0.7) were not offered into the same model, with the more suitable variable being chosen based on missing values, reliability of measurement or biological plausibility. Potential confounders were simultaneously offered into the initial model. Following this, biologically plausible effect measure modification on the multiplicative scale was evaluated by fitting interaction terms as fixed effects. For interaction terms with Wald tests at $P < 0.05$, effect estimates were stratified by the effect-modifying variable and if biologically relevant, were included in the final model. Interaction terms were removed from the model if they had Wald tests at $P > 0.05$ or if they did not add subjective explanatory value to the model. Following this, covariates (potential confounders) were removed from the model one step at a time, and

replaced back into the model if removal changed the effect estimate for SDCT groups by more than 10% (Greenland and Pearce, 2015).

4.3.6.4 Effect of Selective Dry Cow Therapy on Daily Milk Yield and $\log_e$ Somatic Cell Count in the First 120 Days in Milk

The effect of treatment group on monthly test-day $\log_e$ transformed SCC and milk yield was analyzed using linear mixed models. Unconditional effect estimates for the relationship between explanatory variables of interest and outcome variables (i.e. SCC and milk yield) were calculated using linear regression. For multivariable models, random intercepts for cow and herd were used to account for the clustering of tests within cows, and cows within herds. Test DIM was forced into the final model as a fixed effect (levels: 0-20, 21-40, 41-60, 61-80, 81-100, 101-120 DIM). The same potential confounder list and general model building strategy for clinical mastitis and removal from the herd outcomes was used to create final multivariable linear mixed models. Assumptions of homoscedasticity and normality of residuals were evaluated using residual scatter plots and normal quantile-quantile plots. Estimated marginal means (model adjusted means) on the log and linear (i.e. geometric means) scales were used to make comparisons between treatment groups.

4.4 Results

4.4.1 Enrollment

A detailed description of the study herds can be found in Rowe et al. (Submitted-b). At enrollment, cows were randomly allocated to Blanket (n = 429), Culture (n = 432) and Algorithm (n = 414) groups. Median age (skewed distribution) of subjects at enrollment

was 45 months (interquartile range [**IQR**] = 34 – 56), average DIM at dry-off was 325 d (SD = 46) and mean milk yield at the most recent DHIA test prior to dry-off was 27.3 kg (SD = 8.7). The proportion of cows of parity 1, 2 and ≥3 at enrollment was 42%, 30% and 28% and the proportion of cows at enrollment that had experienced at least one clinical mastitis event during the lactation of enrollment was 14%. At enrollment, the quarter-level prevalence of IMI was 25.4% (1078 / 4242), which was composed of non-aureus *Staphylococcus* spp. (14.8%), *Streptococcus* and Strep-like organisms (2.2%), other Gram-positive bacteria (7.9%), Gram-negative bacteria (1.5%), and other pathogens (0.1%). Treatment groups were balanced at enrollment for demographic variables and IMI prevalence, which is described in detail in Rowe et al. (Submitted-b).

4.4.2 Losses to Follow-up and Adverse Events during the Dry Period

Three enrolled cows were retrospectively excluded because of recent (<14d) antibiotic treatments before enrollment. At dry-off (2 days after enrollment), 29 cows were removed from the study because they did not meet the inclusion criteria, mostly due to being found non-pregnant. During the dry period, 11 cows died or were culled from the Blanket (n = 5), Culture (n = 1) and Algorithm (n = 5) groups. No mastitis cases were recorded for culled or dead animals during the dry period. Five of 1211 (0.4%) cows had a recorded case of clinical mastitis during the dry period (Blanket = 1/407, Culture = 1/410, Algorithm = 3/394). An additional 17 and 4 cows were retrospectively excluded from the study, because of short (<30 d) or long (>90 d) dry periods, respectively. In total, 22, 22 and 20 cows from Blanket, Culture and Algorithm groups were lost to follow-up between enrollment and calving, respectively. Consequently, the number of

cows available for post-calving outcomes was Blanket (n = 407), Culture (n = 410) and Algorithm (n = 394).

4.4.3 Effect of Selective Dry Cow Therapy on Risk for Clinical mastitis and Risk for Removal from the Herd during the First 120 Days in Milk

Unconditional RR estimates for 10 potential predictors of 120-day clinical mastitis and removal from the herd risk are shown in Table 4.1. The overall 120-day clinical mastitis risk was 13.0% of cows (157 / 1211). The crude 120-day clinical mastitis risk was similar in Blanket (59 / 407, 14.5%), Culture (50 / 410, 12.2%) and Algorithm (48 / 394, 12.2%) treatment groups. Kaplan-Meier curves indicated that clinical mastitis rates were similar between treatment groups (Figure 4.1; log-rank test at P = 0.5). The final Cox proportional hazards model evaluating the effects of treatment group on hazards of clinical mastitis is shown in Table 4.2. Clinical mastitis HR estimates for Culture and Algorithm groups (ref = Blanket) were 0.82 (95% CI: 0.58, 1.15) and 0.83 (0.63, 1.09), respectively.

The overall 120-day risk of removal from the herd was 10.4% (126 / 1211), which was similar in Blanket (44 / 407, 10.8%), Culture (40 / 410, 9.8%), and Algorithm (42 / 394, 10.7%) treatment groups. Kaplan-Meier curves indicated that the rate of removal from the herd was similar between treatment groups (Figure 4.1; log-rank test at P = 0.8). The final Cox proportional hazards model evaluating the effect of treatment group on hazards of removal from the herd during 1-120 DIM is shown in Table 4.2. Removal from the herd HR estimates for Culture and Algorithm groups (referent: Blanket) were 0.89 (95% CI: 0.60, 1.32) and 0.98 (95% CI: 0.67, 1.43), respectively.

No fixed-effect covariates were included in the final models for clinical mastitis and removal from the herd, as no confounders were identified when using the 10% change in estimates model building approach.

4.4.4 Effect of Selective Dry Cow Therapy on Test-Day Milk Yield and Somatic Cell Counts during 1-120 Days in Milk

Unconditional estimates for potential predictors of test day $\log_e$ SCC and milk yield are shown in Table 4.3. The final linear mixed model for the effect of treatment group on $\log_e$ SCC is shown in Table 4.4. The estimated differences in $\log_e$ SCC for Culture minus Blanket and Algorithm minus Blanket were +0.05 (95% CI: -0.09, 0.18) and +0.07 (95% CI: -0.7, 0.21), respectively. The estimated marginal means for each treatment group on the $\log_e$ scale were Blanket (4.01, 95% CI: 3.85, 4.17), Culture (4.06, 95% CI: 3.90, 4.21) and Algorithm (4.09, 95% CI: 3.92, 4.24). On the linear scale, estimated marginal means (i.e. geometric mean) for SCC (x 1000 cells per ml) were Blanket (55, 95% CI: 47, 65), Culture (58, 95% CI: 49, 68) and Algorithm (59, 95% CI: 50, 69). Estimated marginal means stratified by DIM category are plotted in Figure 4.2. The final model included fixed effects for test DIM, clinical mastitis during the lactation of enrollment and $\log_e$ SCC prior to enrollment. No interaction was observed between treatment group and test DIM ($P > 0.05$). Intraclass correlation coefficients from a random effects model (no fixed effects), for cow and herd were 0.35 and 0.02.

The final linear mixed model for the effect of treatment group on daily milk yield (kg) is shown in Table 4.4. The estimated differences in milk yield for Culture minus Blanket and Algorithm minus Blanket were -0.04 (95% CI: -0.96, 0.87) and -0.92 kg (-1.84, 0.00)

respectively. The estimated marginal means for each treatment group were Blanket (48.7, 95% CI: 46.3, 51.2), Culture (48.7, 95% CI: 46.2, 51.1) and Algorithm (47.8, 95% CI: 45.4, 50.3). Estimated marginal means stratified by test DIM are plotted in Figure 4.2. The final model included fixed effects for test DIM, clinical mastitis during the lactation of enrollment and parity, milk yield and $\log_e$ SCC prior to enrollment. No interaction was observed between treatment group and test DIM ($P > 0.05$). Intraclass correlation coefficients from a random effects model, for cow and herd were 0.25 and 0.09.

4.5 Discussion

4.5.1 Incidences of Clinical Mastitis and Removal from the Herd During the Dry Period were Low in All Treatment Groups

We observed a low incidence (0.9%) of removal from the herd during the dry period (Blanket = 5/407, Culture = 1/409, Algorithm = 5/391). In addition, we observed a low incidence (0.4%) of dry-period clinical mastitis in all treatment groups (Blanket = 1/407, Culture =1/409, Algorithm = 3/391). This incidence may be underestimated, as detection of clinical mastitis during the dry period can be challenging for farm workers. Our finding is inconsistent with some 'failing' SDCT trials which found substantial increases in clinical mastitis incidence for the SDCT group (vs BDCT) during the dry period (Berry and Hillerton, 2002, McDougall, 2010, Scherpenzeel et al., 2014). We hypothesize that our SDCT programs may have succeeded, in part, due to the use of an ITS, whereas the aforementioned trials omitted use of an ITS in their SDCT protocols. It is well documented that ITS, whether used alone or in combination with an intramammary antibiotic at dry off, significantly reduce the risk for new IMI during the dry period (Rabiee and Lean, 2013).

4.5.2 Risk for Clinical mastitis and Removal from the Herd were Similar between Treatment Groups

We found that culture- and algorithm-guided SDCT are unlikely to cause marked increases in clinical mastitis during 1-120 DIM, as evidenced by HR estimates and their 95% confidence intervals being less than or close to 1 (i.e. HR for Culture / Blanket = 0.82, 95% CI: 0.58, 1.15 and Algorithm/Blanket = 0.83, 95% CI: 0.62, 1.09). This finding is consistent with recent SDCT studies, which found comparable proportions of clinical mastitis during early lactation between BDCT and SDCT groups (Bradley et al., 2010, Cameron et al., 2014, Vasquez et al., 2018). In contrast to these recent studies, older SDCT trials that did not use ITS repeatedly found higher clinical mastitis incidences (Berry and Hillerton, 2002, McDougall, 2010, Rajala-Schultz et al., 2011, Scherpenzeel et al., 2014).

Proportions of cows culled or died during 1-120 DIM were also similar between treatment groups in our study. However, the HR estimates were imprecise (HR for Culture/Blanket = 0.89, 95% CI: 0.60, 1.32 and HR for Algorithm/Blanket = 0.98, 95% CI: 0.67, 1.43). For example, our statistical analysis indicated that the hazard rate for removal from the herd under an algorithm-guided SDCT program could be 23% lower or 43% higher than that observed under a BDCT program, which at either extreme, would be of significance to veterinarians and producers. Given the uncertainty in these estimates, we cannot infer what the likely impact of SDCT would be in commercial dairy herds. Very few studies have compared the risk of removal from the herd in cows assigned to SDCT and BDCT. Vasquez et al. (2018) found that among cows classified as ‘low risk’ by their algorithm, not treating with antibiotics was associated with lower odds

of removal from the herd within the first 30 DIM than cows that received antibiotics (OR = 0.83), but that estimate was, like in our study, imprecise (95% CI: 0.40, 1.69). Trials with larger sample sizes or meta-analyses could be used to further investigate the effect of SDCT on risk of removal from the herd.

4.5.3 Somatic Cell Count and Milk Yield were Similar between Treatment Groups

Our analysis also found that the difference in test day $\log_e$ SCC between our treatment groups was negligible (Culture minus Blanket = 0.05, 95% CI: -0.09, 0.18 and Algorithm minus Blanket = 0.07, 95% CI: -0.07, 0.21). Other recent SDCT trials using teat sealants have found similar negligible effects on early lactation SCC (Cameron et al., 2015, Vasquez et al., 2018, McParland et al., 2019). Earlier trials of SDCT protocols not using ITS identified appreciable increases in SCC in early lactation (McDougall, 2010, Scherpenzeel et al., 2014).

The final multivariable model for the effect of SDCT on milk yield lacked precision to make meaningful inferences (Culture minus Blanket = -0.04 kg, 95% CI: -0.96 to 0.87, Algorithm minus Blanket = -0.92, 95% CI: -1.84, 0.00). A recent SDCT trial conducted in Ireland found that cows randomized to an algorithm-guided SDCT protocol had higher average daily milk yield (+0.67 kg) over the course of an entire lactation (McParland et al., 2019). However, most other SDCT trials have found small to negligible impacts on milk yield (Cameron et al., 2015, Vasquez et al., 2018). Meta-analysis could be used to further evaluate the effect on SDCT on milk yield.

4.5.4 Study Strengths and Limitations

4.5.4.1 Internal Validity

A detailed description of the strengths and limitations of this study can be found in Rowe et al. (Submitted-b). Measurement error is a concern for any study. For example, clinical mastitis cases in this study were detected and recorded by farm workers, not study personnel. Consequently, we were not able to guarantee that all clinical mastitis cases recorded in this study were based on the same case definition. However, we made conscious efforts to select herds that detected and recorded clinical mastitis in a way that was consistent with our case definition (i.e. visible abnormalities in the milk). We expect removal from the herd, SCC and milk yield outcomes to be less subjective and thus, less vulnerable to potential measurement error. We expect that masking farm staff to treatment group status would prevent measurement error from impacting treatment groups differently, which minimizes the biasing impact of misclassification. However, as mentioned earlier, it was possible that farm workers on two farms could have known treatment groups, due to treatment records being shared with the farm manager.

Confounding is an unlikely source of bias in our study. Prior analysis demonstrated that our randomization scheme worked well, with no important demographic differences among cows assigned to the three treatment groups (e.g. parity, dry period length, previous lactation milk yield) (Rowe et al., submitted). For our clinical mastitis and removal from the herd models, no covariates were found to change effect estimates by >10% when they were removed from the models, indicating that there was little or no measured confounding. However, this did occur for SCC and milk yield outcomes, which we think is unlikely to be due to confounding, but rather because of very small effect

estimates, which has been shown to increase the probability of variables being included in the final model when using a 10% change-in-estimates approach (Lee, 2014). This is supported by the small effect estimates, and the balance of potential confounders among the three treatment groups at baseline (Rowe et al. Submitted).

4.5.4.2 External Validity

We believe that our research is relevant to a large proportion of American dairy farmers, because cows were enrolled from seven herds located in the west, midwest, and northeast dairy farming regions of the U.S. Furthermore, study herds used similar management practices and had similar prevalences of IMI at dry-off to other herds recently studied in the U.S. (Rowe et al., 2019b, Rowe et al., In-Press-b). However, readers should consider the herd enrollment criteria that were used, which may have favored selection of larger, better managed herds.

The use of study technicians, and not farm staff to administer intramammary treatments serves to improve internal validity, as it means that cows across herds received treatments in the same, standardized manner. However, this could potentially limit the external validity for farms where staff fail to administer treatments according to the manufacturer's recommendation. Consequently, we strongly recommend that producers implementing SDCT take special care when administering intramammary treatments.

Another limitation to the external validity of this study is that long-term effects were not assessed. It is possible that potential negative impacts of SDCT may take several lactations before they can be clearly observed. We chose to focus on early lactation

health events and production because dry-period IMI have the greatest impact during this time.

4.5.5 Future Research

Further work in this multi-site project will investigate: 1) test characteristics of rapid culture and our algorithm; 2) the economic impact of SDCT; and 3) the possible microbiome impact of SDCT. Further research of SDCT as implemented by farm staff (and not research technicians) would further evaluate the suitability of SDCT on U.S. dairy farms. In addition, more work is needed to identify herd factors that may be used to predict suitability for adoption of SDCT.

4.5.6 Conclusion

In a multi-site, randomized, natural exposure trial, culture- and algorithm-guided SDCT each reduced antibiotic use without having a negative impact on cow health and performance, including risk for clinical mastitis, risk for removal from the herd, and DHIA measures of SCC and milk yield during the first 120 DIM. These findings indicate that SDCT can be successfully implemented on appropriate commercial dairy herds in the U.S.

4.6 Declaration of competing interest

The Minnesota Easy® 4Cast® plate is manufactured by the University of Minnesota (St. Paul, MN). However, the study investigators have no financial interest in the sale of this plate.

4.7 Authors roles

SM. Rowe was involved in local and multi-site coordination, fieldwork, laboratory work, data management, statistical analysis and manuscript preparation. SM. Godden was involved in study conceptualization, herd recruitment in MN and WI, supervision of fieldwork and manuscript editing. PJ. Gorden and A. Lago were involved in study conceptualization, herd recruitment, local site coordination, fieldwork and manuscript editing. DV. Nydam was involved in study conceptualization, NY herd recruitment, local site coordination, and manuscript editing. AK. Vasquez was involved in fieldwork, local site coordination and manuscript editing. E. Royster was involved in study conceptualization and manuscript editing. J. Timmerman was involved in laboratory work and manuscript editing. MJ. Thomas was involved in local site coordination, fieldwork and manuscript editing.

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Minnesota (Kelli Bowman, Joshua Brown, Pedro Paulo Cecillio Ferro, Chandra Dahike, Kaylan Risacher and Victor Moro Taveira), New York (Lauren Pitman and Michaela Thomas).

Table 4.1: Unconditional analysis for possible predictors of clinical mastitis and removal from the herd during the first 120 days in milk.

	Clinical mastitis (n at risk = 1211)		Removal from the herd (n at risk = 1211)	
	Risk	**Risk Ratio (95% CI)**	**Risk**	**Risk Ratio (95% CI)**
All cows	13.0%		10.4%	
Treatment group				
Blanket	14.5%	Ref	10.8%	Ref
Culture	12.2%	0.84 (0.59, 1.20)	10.7%	0.99 (0.66, 1.47)
Algorithm	12.2%	0.84 (0.59, 1.19)	9.8%	0.90 (0.60, 1.35)
Age (years)		1.09 (0.97, 1.24)		1.41 (1.25, 1.6)
Parity at enrollment				
1	10.8%	Ref	5.7%	Ref
2	14.3%	1.32 (0.93, 1.89)	9.1%	1.59 (0.99, 2.59)
≥3	14.8%	1.38 (0.96, 1.97)	19.0%	3.34 (2.23, 5.15)
DIM at dry-off (per 10d)		0.99 (0.95, 1.02)		1.01 (0.98, 1.05)
Milk yield at last test (kg)		0.99 (0.98, 1.01)		0.98 (0.96, 0.99)
SCC at last test ($\log_e$[cells/1000] per ml)		1.09 (0.96, 1.22)		1.26 (1.11, 1.42)
Peak SCC during lactation of enrollment ($\log_e$[cells/1000] per ml)		1.04 (0.92, 1.16)		1.26 (1.11, 1.42)
SCC at last test				
<200,000	12.3%	Ref	9.2%	Ref
≥200,000	15.4%	1.25 (0.89, 1.72)	14.6%	1.58 (1.10, 2.23)
Clinical mastitis during lactation of enrollment				
No cases	12.2%	Ref	9.6%	Ref
≥1 cases	17.6%	1.44 (0.97, 2.04)	15.8%	1.65 (1.08, 2.41)
Dry period length (per 10d)		0.84 (0.70, 1.01)		0.76 (0.62, 0.94)

[1]Risk ratio estimates are per 10 days. For example RR of clinical mastitis = 0.99, means that risk of clinical mastitis is 1% less for every additional 10 days in milk at enrollment.

Table 4.2: Results of final cox proportional hazards regression models describing the effect of two selective dry cow therapy programs when compared with blanket dry cow therapy, on risk for clinical mastitis and removal from the herd during the first 120 days of lactation.

	Crude 120d risk	Hazard ratio[1]	Robust SE[2]	95% CI of Hazard ratio
Clinical mastitis[3]				
Blanket	14.5%	Ref		
Culture	12.5%	0.82	0.18	0.58, 1.15
Algorithm	12.1%	0.83	0.14	0.63, 1.09
Removal from the herd[3]				
Blanket	10.7%	Ref		
Culture	9.6%	0.89	0.20	0.60, 1.32
Algorithm	10.6%	0.98	0.19	0.67, 1.43

[1]Hazard ratio estimates were derived from Cox proportional hazards regression.
[2]Standard errors determined using a robust sandwich estimator to account for clustering of cows within herds.
[3]Backwards selection resulted in no fixed effect covariates being included in the final model.

Table 4.3: Univariable analysis for possible predictors of log_e somatic cell count and milk yield during the first 120 days of lactation

	Log somatic cell count		Milk yield (kg/d)	
	Mean	**Difference**	**Mean**	**Difference**
All cow-tests	3.92		4.91	
Treatment group				
Blanket	3.91	Ref	49.2	Ref
Culture	3.93	+0.02 (-0.08, 0.13)	48.6	-0.62 (-1.42, 0.19)
Algorithm	3.93	+0.02 (-0.08, 0.12)	49.5	+0.33 (-0.47, 1.12)
Age (years)		+0.12 (0.09, 0.15)		0.65 (0.44, 0.86)
Parity at enrollment				
1	3.75	Ref	47.9	Ref
2	3.97	+0.22 (0.12, 0.32)	49.6	+1.69 (0.92, 2.46)
≥3	4.16	+0.41 (0.30, 0.51)	50.5	+2.53 (1.73, 3.33)
DIM at dry-off (per 10 d)[1]		0.00 (-0.01, 0.01)		+0.12 (0.05, 0.19)
Milk yield at last test (kg)		-0.01 (-0.01, 0.00)		+0.22 (0.18, 0.26)
SCC at last test (log_e[cells/1000] per ml)		+0.19 (0.15, 0.22)		+0.22 (-0.04, 0.49)
Peak SCC during lactation of enrollment (log_e[cells/1000] per ml)		+0.22 (0.19, 0.25)		+0.47 (0.21, 0.73)
SCC at last test				
<200,000	3.83	Ref	49.1	Ref
≥200,000	4.25	+0.42 (0.32, 0.52)	49.1	-0.03 (-0.82, 0.75)
Clinical mastitis during lactation of enrollment				
No cases	3.86	Ref	49.1	Ref
≥1 cases	4.35	+0.50 (0.37, 0.62)	49.4	+0.34 (-0.62, 1.31)
Dry period length (per 10 d)		-0.04 (-0.10, 0.01)		+0.70 (0.29, 1.12)

[1]Difference in means estimates are per every 10 days. For example an estimate of +0.12, means that milk yield increases by 0.12 kg for every additional 10 days in milk at enrollment.

Table 4.4: Results of final multivariable regression models estimating the effect of two selective dry cow therapy programs compared with blanket dry cow therapy on log_e somatic cell count and milk yield during the first 120 days of lactation.

	Adjusted mean (SE)	Difference	95% CI of difference
Log_e Somatic cell count (log_e[cells/1000] per ml)[2]			
Blanket	4.01 (0.08)	Ref	
Culture	4.06 (0.08)	+0.05	-0.09, 0.18
Algorithm	4.08 (0.08)	+0.07	-0.07, 0.21
Average daily milk yield (kg/day)[3]			
Blanket	48.7 (1.25)	Ref	
Culture	48.7 (1.25)	-0.04	-0.96, 0.87
Algorithm	47.8 (1.25)	-0.92	-1.84, 0.00

[1]Estimated marginal means / least squares means.
[2]Fixed effect covariates included: Days in milk at herd test, clinical mastitis during lactation of enrollment (Y / N), and somatic cell count at last herd test.
[3]Fixed effect covariates included: Days in milk at herd test, clinical mastitis during lactation of enrollment (Y / N), parity at enrollment, somatic cell count at last herd test, and milk yield at last herd test.
Intraclass coefficients for log somatic cell count for cow and herd were 0.35 and 0.02, respectively.
Intraclass coefficients for milk yield for cow and herd were 0.25 and 0.09, respectively.

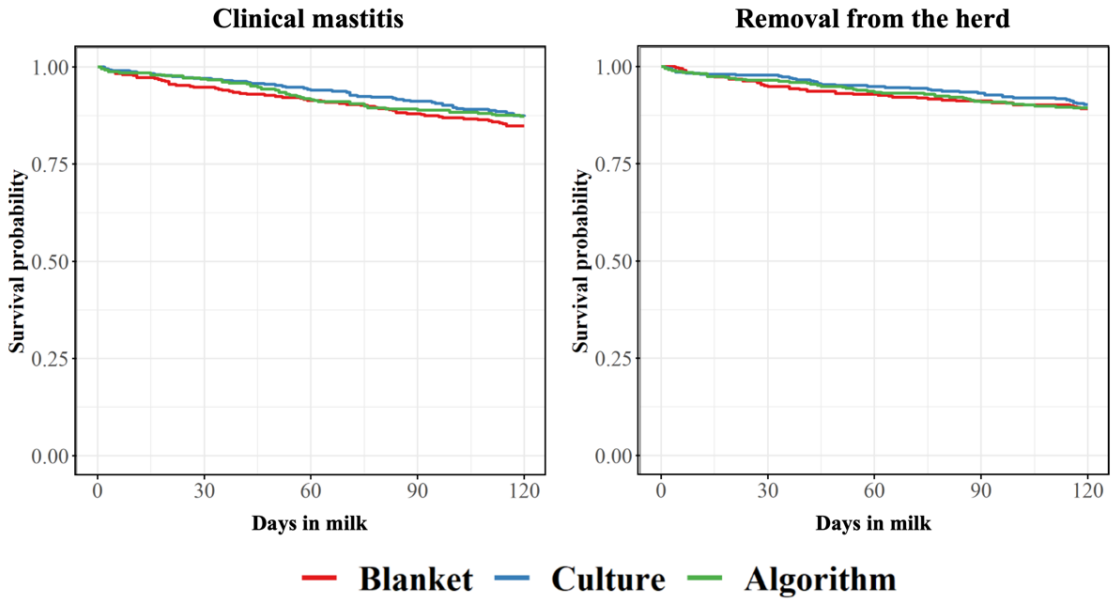

Figure 4.1: Kaplan-Meier curves showing the incidence of clinical mastitis and removal from the herd over the first 120 days of lactation for cows in a blanket dry cow therapy or one of two selective dry cow therapy programs. Log-rank test for clinical mastitis and removal from the herd were P = 0.5 and P = 0.8, respectively.

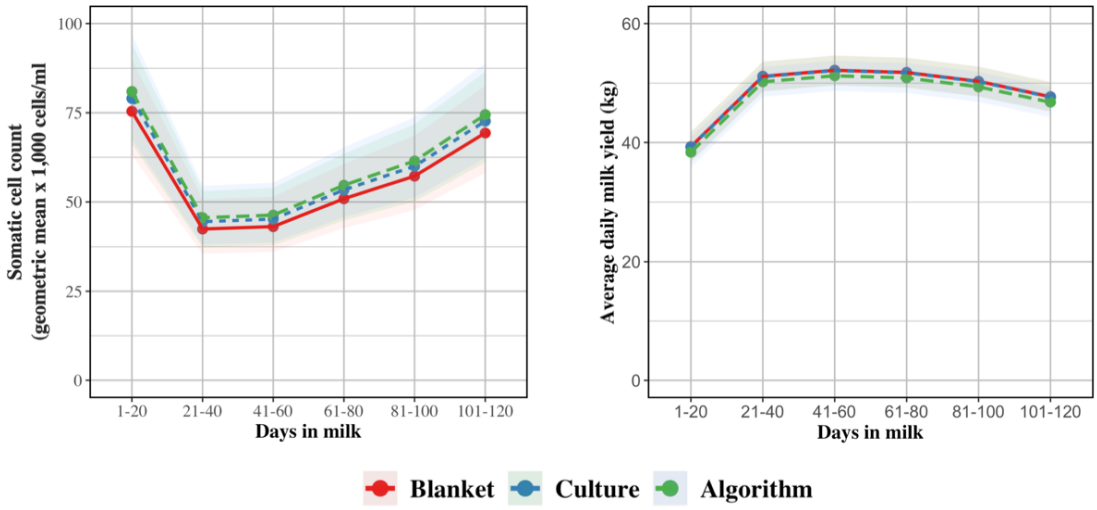

Figure 4.2: Estimated marginal means of somatic cell count and milk yield during the first 120 days of lactation for cows receiving blanket dry cow therapy or one of two selective dry cow therapy strategies. 95% confidence intervals are indicated by colored shaded areas. No statistical interaction was observed between treatment group and days in milk at herd test, which is why curves are parallel.

5 CHAPTER FIVE: Evaluation of Rapid Culture, a Predictive Algorithm, Esterase Somatic Cell Count and Lactate Dehydrogenase to Detect Intramammary Infection in Quarters of Dairy Cows at Dry-Off

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5.1 Summary

Our objective was to compare four tests to standard milk culture followed by MALDI-ToF in quarters of cows at dry-off. Cows (n=432) were randomly selected from seven U.S. dairy herds already participating in a multi-site clinical trial in summer 2018. Aseptic foremilk samples were collected from quarters (n=1728) two days prior to dry-off, and subjected to index and reference tests. The four index tests included rapid culture, a predictive algorithm, an esterase strip test measuring somatic cell count (**SCC**) and a cow-side lactate dehydrogenase (**LDH**) test. Rapid culture was performed by inoculating quarter milk samples onto a commercial rapid culture plate. Plates were evaluated by technicians after 30-40 hours of incubation at 37 ± 2°C. Quarters were classified as infected if any bacterial growth was observed. For the algorithm test method, all quarters were classified as infected if the cow met any of the following criteria: 1) any Dairy Herd Improvement Association (**DHIA**) test with a SCC > 200,000 cells / ml during the current lactation; 2) two or more clinical mastitis cases during the current lactation; or 3) one clinical mastitis case in the 14 days prior to enrollment. Esterase-SCC and cow-side LDH tests involved adding milk to the test strip and reading for color changes. For esterase-SCC and cow-side LDH tests, low (≥250 cells / ml and ≥100 U / L)

and high (≥500 cells / ml and ≥200 U / L) thresholds were used to classify quarters as infected or not. Composite samples (4 x 2ml quarter-milk samples commingled) were also tested for rapid culture, esterase-SCC and cow-side LDH tests, such that if a composite sample was positive, then all quarters contributing to that sample were classified as infected. The reference test was traditional aerobic culture conducted in an accredited laboratory using MALDI-ToF for identification of isolates. Traditional culture was only conducted on quarter-milk samples, and consequently, IMI was always considered at the quarter-level. Unconditional logistic regression was used to estimate sensitivity (**SE**), specificity (**SP**), apparent prevalence, positive predictive values (**PPV**) and negative predictive values (**NPV**) for each index test. Cohen's Kappa (κ) was used to measure agreement between tests. Algorithm, esterase-SCC and cow-side LDH tests had poor agreement with the reference test (κ ranging from 0.01 to 0.12), while rapid culture had fair agreement (κ = 0.28). No test had concurrently high SE and SP. Negative predictive values were moderate to high for all tests.

5.2 Introduction

Dairy producers need practical ways to reduce antibiotic use where possible, in order to address consumer concerns about antimicrobial stewardship in food animal production. Selective dry cow therapy (**SDCT**) reduces antibiotic use by screening cows or quarters at the time of dry-off to identify candidates for antibiotic treatment (dry cow therapy). Clinical trials have found that implementing SDCT can successfully reduce quarter-level antibiotic use by 21 to 58% without compromising cow health in the subsequent lactation (Cameron et al., 2014, Kabera et al., 2019, Rowe et al., In-Press-a, Submitted-b). Furthermore, overall reductions in antibiotic use at the national level have been recently

observed following industry-wide adoption of SDCT in the Netherlands (Vanhoudt et al., 2018). Despite the potential of SDCT to improve antibiotic stewardship, most U.S. dairy producers (80%) continue to practice blanket dry cow therapy (USDA-NAHMS, 2014), which involves antibiotic treatment of all cows at the end of lactation, irrespective of an individual cow's IMI status. This low level of uptake of SDCT programs among U.S. commercial farms may be due to perceived risks to cow health and logistical challenges surrounding the implementation of SDCT programs. The former concerns are justified, given that some early SDCT programs resulted in impaired udder health in the subsequent lactation (McDougall, 2010, Scherpenzeel et al., 2014). However, several more recent SDCT programs have been successful in reducing antibiotic use while maintaining future cow health and performance (Cameron et al., 2014, Rowe et al., Submitted-b). Based on the research to date, we believe that in order to be successful, SDCT programs will use a teat sealant to protect untreated quarters from new IMI during the dry period, and will adopt a sufficiently accurate screening test to identify infected cows or quarters in need of antibiotic treatment at dry-off. Consequently, the ideal screening test for a SDCT program would be cost-effective, easy to implement, and be able to accurately identify quarters that require antibiotic treatment and those that do not. In theory, identification of IMI by direct tests, such as milk culture, is the preferred method for selecting quarters of cows to receive antibiotic dry cow therapy, as IMI at dry-off are associated with reduced udder health in the subsequent lactation (Green et al., 2002) and because demonstration of infection is a recommended prerequisite for antibiotic therapy in veterinary medicine (WHO, 2017). However, bacterial culture at a commercial reference laboratory is often impractical and costly for producers. To date,

rapid culture systems conducted on farm or in a local veterinary laboratory and predictive algorithms have been clinically evaluated more than any other approach, and both were used in SDCT protocols that reduced antibiotic use at dry-off without any associated negative effects on udder health (Bradley et al., 2011, Cameron et al., 2014, Patel et al., 2017, Vasquez et al., 2018, Rowe et al., In-Press-a, Submitted-b).

Despite the aforementioned success in rapid culture- and algorithm-guided SDCT, some producers may not adopt their use, especially those unable or unwilling to conduct monthly DHIA testing, those unable or unwilling to set up a rapid culture laboratory on farm, or those who do not have access to rapid culture through their veterinarian's practice. Consequently, other potential screening tools should be investigated. A recent study showed that a commercial somatic cell count (**SCC**) esterase strip test (PortaSCC®; PortaCheck, Moorestown, NJ) had a SE and SP of 0.78 and 0.73, respectively for IMI at dry-off (Kandeel et al., 2019). In addition, recent studies have shown that milk lactate dehydrogenase (**LDH**) concentrations are elevated in quarters with IMI (Hernández-Castellano et al., 2017, Khatun et al., 2019), and a cow-side LDH strip test has been recently developed (UdderCheck®, PortaCheck, Moorestown, NJ). Rapid testing of quarter-milk samples for SCC or LDH can be conducted on the day of dry-off without the use of an on-site laboratory, and therefore could be a viable alternative to rapid culture and predictive algorithms. However, these tests require further validation, as the favorable characteristics of esterase-SCC observed in the Kandeel et al. (2019) study were described in a relatively small sample of quarters (n=380) from a single farm. Furthermore, the aforementioned cow-side LDH test has not been evaluated

in field conditions. Therefore, research is needed to evaluate this test in late lactation cows. The objective of this multi-site study was to compare the four screening tests (rapid culture, a predictive algorithm, esterase SCC and cow-side LDH) to culture/MALDI of quarters in dairy cows at dry-off.

5.3 Materials and Methods

This cross-sectional study was conducted as part of a multi-site randomized clinical trial evaluating the effect of two SDCT programs, a rapid culture-guided program or an algorithm-guided program, on antibiotic use, IMI dynamics during the dry period (Rowe et al., Submitted-b) and post-calving health (Rowe et al., In-Press-a). The objectives reported in this manuscript were part of the original study design, however, no formal sample size calculations were performed for this particular objective. Details of the sample size calculations for IMI dynamics during the dry period can be found in Rowe et al. (Submitted-b). The Standards for Reporting of Diagnostic Accuracy Studies (STARD) statement was used in the reporting of this manuscript (Cohen et al., 2016).

5.3.1 Study Herds

Seven herds were recruited from five states as a convenience sample (California = 2 herds; Iowa = 1 herd; Minnesota = 1 herd; New York = 2 herds; Wisconsin = 1 herd), with cows enrolled from May to July, 2018. Herds were selected because they had a working relationship with local project collaborators, had a herd size sufficiently large to dry off at least 15 cows per week, had an average bulk milk SCC less than 250,000 cells / ml during the previous 12 months, were on a monthly DHIA testing schedule (i.e. individual cow SCC measurements), and consistently recorded all clinical mastitis events.

Herds were not screened for Mycoplasma IMI at herd enrollment. Herd participation was voluntary.

5.3.2 Subject Enrollment

Study technicians visited herds each week to enroll cows two days prior to the planned dry-off date. Cows were eligible for enrollment if they had 4 functional quarters, an expected dry period length of 30 to 90 days, no recent antibiotic or anti-inflammatory treatment (within 14 d), no clinical mastitis (i.e. no visible abnormalities present in foremilk or heat, erythema or pain detected upon palpation of the udder), were not lame (> 3/5; Sprecher et al. (1997)), or in poor body condition (< 2/5; Edmonson et al. (1989)). At each weekly enrollment day, cows were block-randomized to one of three groups by study technicians: blanket dry cow therapy, culture-guided SDCT, or algorithm-guided SDCT. Following enrollment, duplicate, aseptic quarter-milk samples were collected from cows according to NMC guidelines (NMC, 2017). Briefly, after farm staff performed their usual pre-milking teat disinfection routine, study technicians, who were wearing clean disposable gloves, scrubbed teat ends with 70% isopropyl alcohol-soaked gauze swabs, discarded three squirts of foremilk and sampled approximately 20-30 ml of milk into sterile 60-ml vials. Samples were immediately chilled on ice and transported back to the site laboratory. The order of groups within blocks was determined using a random number generator. Only quarter milk samples from cows in the culture-guided SDCT group were recruited into this particular test evaluation study.

5.3.3 Index Tests

The four evaluated index tests were rapid culture, a predictive algorithm, esterase-SCC ("PortaSCC"), and cow-side LDH ("UdderCheck"). Technicians were blinded to the reference test results at the time that index tests were performed. Definitions for each test are summarized in Table 5.1. Index tests were conducted by study technicians at the four study sites (California, Iowa, Minnesota, and New York) using a standardized protocol. Composite samples for each cow were created at the study site (i.e. 4 x 2ml quarter-milk samples per cow). Testing was performed on quarter and composite samples for all tests except for algorithm. The algorithm results were grouped with composite tests, because the algorithm included DHIA composite SCC results. Test results for quarter and composite samples were recorded at the quarter-level for comparison to the reference test which was performed at the quarter level only. When composite samples were tested, all quarters within a single cow received the same status. Testing of milk samples commenced within 6 hours of sample collection.

Rapid culture involved inoculation of quarter and composite samples (~0.1 ml) onto a commercial rapid culture system (Minnesota Easy® 4Cast® plate, University of Minnesota, St Paul, MN) using sterile cotton-tip swabs and incubation of each plate at 37 ± 2°C for 30-40hrs. Culture plates were read by study technicians ("Rapid culture: technician") and a worker from each farm ("Rapid culture: farmer") who were blinded to each others' results. Quarter or composite samples with any growth, including those with patterns suggestive of contamination (i.e. numerous independent isolates) were classified as infected.

For PortaSCC and UdderCheck tests, quarter and composite milk samples were allowed to warm to room temperature and were mixed by gentle inversion before testing. The PortaSCC test was conducted by adding 40µl of milk and 3 drops of activator solution to the test strip and reading 45 to 60 minutes later. The color of the test strip was visually compared to a reference chart to estimate the somatic cell count (**SCC;** x 1,000 cells/mL) at <100, 250, 500, 750, 1,500, or >3,000. The UdderCheck test was conducted by adding 40µl of milk to the test strip and reading four minutes later. The color of the test strip was visually compared to a reference chart to estimate LDH concentration (U/L) at <100, 100 – 200, 200 – 500, and > 500. PortaSCC was dichotomized using two different cut-points: 250 and 500, and UdderCheck was also dichotomized using two different cut-points at 100 and 200, as outlined in Table 5.1. After testing, milk samples were stored -20°C.

For the algorithm method, health records for each cow were later extracted from the farm computer (DC305, Valley Ag Software, Tulare, CA and PCDART, Dairy Records Management Systems, Raleigh, NC) and used in the predictive algorithm. Quarters were classified as infected if the cow met any of the following criteria: ≥2 cases of clinical mastitis during the current lactation, any clinical mastitis during the 14 d prior to dry-off, or any DHIA test-day SCC > 200,000 cells/ml during lactation. If the cow met no criteria, then all quarters were classified as uninfected.

5.3.4 Reference Test: Laboratory Culture

Our objective was to estimate the ability of our index tests to detect IMI at dry-off. Consequently, laboratory milk culture was chosen as the reference test, because it is a widely-accepted method for identifying IMI, and research has been conducted to describe

its reliability as a measure of IMI (Dohoo et al., 2011a, Dohoo et al., 2011b). We estimate that the overall SE and SP of our reference test to be approximately 0.65 and 0.95 respectively, based on research by Dohoo et al. (2011b). The strengths and limitations of using milk culture as a reference test is addressed in the discussion.

Laboratory culture was conducted by experienced lab staff on quarter-milk samples only, and not on the composite samples used with the index tests. After quarter-milk samples were subjected to index tests, they were frozen at -20°C for at least 24 hours before being used to determine 'true' IMI status via aerobic culture. Milk culture was completed at the regional lab (Minnesota, New York, Iowa, California) by laboratory personnel using a common standard operating procedure. On the day of testing, milk samples were thawed at room temperature, homogenized by gentle inversion, and plated onto trypticase soy agar with 5% sheep blood. Agar plates were inoculated with one loop-full (approximately 10µl) of sample, using a sterile disposable plastic loop and incubated in aerobic conditions at 37 ± 2°C for 42-48 hours, after which, plates were interpreted and colony counts recorded by an experienced technician. Only one milk sample from each quarter was cultured, unless the first sample was contaminated, in which case the duplicate sample was cultured. Samples were classified as contaminated if three or more distinct microbial isolates were recovered.

At the regional labs in Minnesota, New York, and Iowa, all isolates were identified using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Microflex; Bruker Daltonics Inc, Billerica, MA). Peaks produced by each isolate were analyzed by the MALDI-TOF Biotyper reference library. The confidence

level for each diagnosis reported by the software was used in the following fashion: >2.0, species level diagnosis recorded; 1.8 – 2, genus level diagnosis recorded; <1.8, MALDI-TOF diagnosis not recorded and traditional identification methods used. Traditional identification methods included colony morphology, catalase reaction, Gram-stain, and cytology. Because the California lab did not have MALDI-TOF available for isolate identification, isolates identified here were stored in glycerol vials at -80°C before submitting them to the Minnesota laboratory. Isolates sent to Minnesota were cultured on Trypticase Soy Agar with 5% Sheep Blood in aerobic conditions and incubated at 37 ± 2°C for 24 hours before being identified using the same methods described earlier.

All-IMI cases were defined as 'significant growth' of any pathogen. Non-aureus *Staphylococcus spp*. (**NAS**) isolates with less than 2 colonies (<200 CFU/ml) and *Bacillus spp*. isolates with less than 5 colonies (<500 CFU/ml) were reclassified as 'non-significant growth' and the quarter considered uninfected (Dohoo et al., 2011b, Johnson et al., 2016). This case definition was used to reduce the risk of misclassification bias when comparing dry-period IMI dynamics between treatment groups in Rowe et al. (Submitted-b).

The ability of tests to predict IMI caused by major pathogens ("Major-IMI") was also investigated, as some mastitis researchers have suggested that antibiotic treatment may only be necessary in major-IMI infected quarters (Pantoja et al., 2009, Bradley et al., 2018, Lipkens et al., 2019), though this hypothesis requires further study. In our analysis, Major-IMI was defined as an IMI caused by *Staphylococcus aureus, Streptococcus* spp., and Strep-like organisms *(i.e. Aerococcus* spp., *Enterococcus* spp*., Lactococcus* spp., *Streptococcus* spp.), coliforms, *Trueperella pyogenes,* and yeast spp. (Gohary and

McDougall, 2018, Lipkens et al., 2019). Quarters infected with non-major pathogens were classified as not infected (i.e. Major-IMI = 0). Laboratory staff conducting the reference test milk cultures were blinded to index test results.

5.3.5 Estimation of Test Characteristics

Quarters without a full set of test results were excluded from analysis. The true prevalence of IMI was calculated as the proportion of quarters testing positive using the reference test. The apparent prevalence was calculated as the proportion of quarters that were test-positive under the index test of interest. Agreement between all tests was calculated using Cohen's Kappa. Estimation of test SE (#test-positives / #true positives), SP (#test-negatives / #true-negatives), PPV (#true-positives / #test-positives), and NPV (#true-negatives / #test-negatives) were conducted using univariable logistic regression models and estimated marginal means, which is equivalent to tabular methods. Youden's index values were calculated as SE + SP – 1. Test characteristics reported for composite-sample testing strategies indicate the ability of a composite test to classify infection status at the quarter-level (i.e. no 'cow-level' definition of All-IMI or Major-IMI was used). Generalized estimating equations (GEE) with binomial family and logit link were used to account for the clustering of quarters within cows and cows within herds, by specifying clustering at the highest level (i.e. herd) and using an 'independence' working covariance structure (Miglioretti and Heagerty, 2006). Given that estimates and 95% CI were very similar for the univariable logistic and GEE models, we have only reported the results from the univariable logistic regression models. All analyses were conducted using the R Statistical Programming Environment (R Core Team, 2018).

5.3.6 Bias Analysis

As a secondary objective, quantitative bias analysis was conducted to evaluate potential misclassification bias due to imperfect measurement of IMI by the reference test (Lash et al., 2011). Bias-adjusted 2 x 2 frequency tables were imputed using the formulae shown in Figure 5.1, with SE and SP for the reference test set at 0.65 and 0.95 for All-IMI and 0.8 and 1.0 for Major-IMI, respectively. These SE and SP assumptions were derived from a weighted average of pathogen-specific estimates reported in Dohoo et al. (2011b). Bias-adjusted 2 x 2 tables were used to calculate bias-adjusted test characteristics. Bias analysis was only conducted for testing of quarter-milk samples with rapid culture, esterase SCC, and esterase LDH, as well as for algorithm, which was considered as a composite sample test.

5.4 Results

5.4.1 Enrollment

All seven herds were predominantly Holstein cows, except for herd 3, which was predominantly Holstein-Jersey cross. Herd size ranged from 850 to 5,700 milking cows and bulk milk SCC prior to enrollment ranged from 90,000 to 230,000 cells / ml. Median age at enrollment (skewed distribution) was 44 months (interquartile range [**IQR**] = 34 – 57), mean DIM at dry-off (normally distributed) was 328 (SD = 47), and mean milk yield at the most recent DHIA test prior to dry-off (normally distributed) was 27.9 kg (SD = 8.8). The proportion of cows of parity 1, 2, and ≥3 at enrollment was 46, 26, and 28%. A more detailed description of enrolled cows and herds at baseline can be found in Rowe et al. (Submitted-b). Figure 5.2 shows the flow of events from enrollment to the final dataset. Of the initial 1,728 quarters from 432 randomly selected cows, 304 quarters were excluded from analysis due to either failure of the cow to meet inclusion criteria (n=40), missing index test information (n=178), or contamination identified at laboratory milk culture (n=86).

5.4.2 Prevalence of Intramammary Infection

According to our reference test, the true prevalence of All-IMI at the quarter-level was 25.4% (361 / 1424, Table 5.2). Infections were predominantly caused by NAS (16.3%, 232 / 1424), *Corynebacterium spp*. (3.4%, 49 / 1424), *Streptococcus* sp. and strep-like organisms (2.2%, 32 / 1424), and *Micrococcus spp*. (1.5%, 22 / 1424). Infections caused by Gram-negative pathogens were rare (0.8%, 11/ 1424). The prevalence of Major-IMI was 2.8% (40 / 1424), which was mostly *Streptococcus* sp. and Strep-like organisms (32 / 40).

5.4.3 Agreement between Tests

Frequency tables for each index test vs the reference test (equivalent to 2 x 2 contingency tables) are summarized in Table 5.3. Cohen's Kappa values for the agreement between all tests are shown as a matrix in Figure 5.3. Kappa values comparing the four index tests to the reference test (All-IMI) ranged from 0.01 (UdderCheck) to 0.28 (rapid culture: technician), indicating a range of poor to fair agreement. Agreement between technician and farmer-read rapid culture was substantial ($\kappa = 0.73$). Moderate agreement was observed between UdderCheck ≥ 200 and PortaSCC ≥ 500 ($\kappa = 0.53$) test results.

5.4.4 Index Test Characteristics

When using quarter-milk samples, the apparent prevalences, or proportion of quarters testing positive to each index test, were rapid culture: technician (0.47), rapid culture: farmer (0.48), PortaSCC ≥ 250 (0.81), PortaSCC ≥ 500 (0.30), UdderCheck ≥ 100 (0.74), and Uddercheck ≥ 200 (0.30). When using composite milk samples, the proportion of quarters testing positive were algorithm (0.56), rapid culture: technician (0.56), rapid culture: farmer (0.52), PortaSCC ≥ 250 (0.88), PortaSCC ≥ 500 (0.37), UdderCheck ≥ 100 (0.78), and Uddercheck ≥ 200 (0.31). The expected apparent prevalence values at different true prevalences are shown in Figure 5.4C.

Table 5.4 and Table 5.5 summarize test characteristics when using quarter-samples for identification of All-IMI sand Major-IMI status respectively, whereas Table 5.6 and Table 5.7 summarize findings when using composite samples for index tests. Across all testing approaches, no tests simultaneously had a high SE and SP, evidenced by

Youden's index values ranging from 0.01 to 0.36. In general, test characteristics were numerically better for rapid culture than the other testing approaches. Rapid culture performed slightly better for prediction of All-IMI than Major-IMI status, while the opposite was observed for algorithm. It is worth noting that sensitivity was moderate to high for PortaSCC ≥ 250 (0.91) and UdderCheck ≥ 100 (0.77), but specificity was very low (0.20 and 0.26), resulting in high apparent prevalences (0.81 and 0.74). Negative predictive value ranges for All- and Major-IMI ranged from 0.75 to 0.87 and 0.94 to 0.97, respectively. Estimates of NPV values declined with increasing prevalence more rapidly for UdderCheck and algorithm tests (Figure 5.4A). Conversely, estimates of PPV increased with increasing prevalence for all tests (Figure 5.4B). However, at all prevalence levels, the PPV was higher for rapid culture than for other tests.

5.4.5 Quantitative Bias Analysis

Bias-adjusted test characteristics for prediction of All-IMI and Major-IMI can be found in Table 5.8 and Table 5.9. Adjustment caused SE to increase for rapid culture: technician (0.73 to 0.79) and rapid culture: farmer (0.72 to 0.78), and slightly for algorithm (0.66 to 0.68) and PortaSCC ≥ 250 (0.88 to 0.91). Adjustment did not change estimates for the UdderCheck test. Bias-adjusted NPV were lower for algorithm (0.75), PortaSCC ≥ 500 (0.72), UdderCheck ≥ 100 (0.69), and UdderCheck ≥ 200 (0.67). Bias-adjusted test characteristics for prediction of Major-IMI were very similar to unadjusted estimates (Table 5.9).

5.5 Discussion

The objective of this study was to compare four index tests to standard milk culture followed by MALDI-ToF to detect IMI in quarters of cows at dry-off, as IMI are considered to be an appropriate target for antibiotic treatment. In the context of SDCT, the SE and SP of a screening test indicates the probability of infected and uninfected quarters being treated and not-treated, respectively. Apparent prevalence and NPV are also useful test characteristics, as they provide an evaluation of the test performance at a given prevalence of disease, which was 25.4% and 2.8% for All- and Major-IMI in this study. Specifically, the apparent prevalence gives an indication of the proportion of quarters that would receive antibiotic treatment under a given SDCT program, whereas the NPV indicates the risk of test-negative (i.e. untreated) quarters being uninfected. Thus, the NPV can be used to estimate the potential for negative udder health impacts caused by failing to treat a truly infected quarter. Interpretation of the test characteristics that serve as a proxy for udder health risks (SE / NPV) is somewhat subjective, as it is not clear what critical levels are required for a SDCT program to avoid negative udder health impacts. Furthermore, we suggest that the reader consider that the NPV for a test that detects no IMI (equivalent to no antibiotic treatment at dry-off) is equal to 1 – prevalence, which in this study equates to 0.75 and 0.97 for All- and Major-pathogens, respectively. This means that under the conditions of this study, it is impossible for any test to have a NPV less than 0.75 and 0.97 for All- and Major-IMI.

The relative importance of these test characteristics will depend on 1) the producer's motivation to reduce antibiotic use at dry-off, which requires high SP and low apparent

prevalence and 2) their motivation to minimize the number of untreated IMI at dry-off, which requires high SE and NPV. The financial and logistical features for each index test are also important factors to consider when making decisions around screening test strategies. Below, we offer a commentary about the performance for each of the tests evaluated in this study, while also considering practical implications.

5.5.1 Algorithm

The predictive algorithm in this study had poor agreement with our reference test (κ = 0.09) for the prediction of All-IMI status, which is evidenced by relatively low SE (0.66) and SP (0.47) estimates. Other studies have also demonstrated the limitations of algorithms for detecting IMI caused by all pathogens at dry-off (Torres et al., 2008, Pantoja et al., 2009, Gohary and McDougall, 2018). Sensitivity increased from 0.66 to 0.72 when only major pathogens were considered, which in this study was similar to the SE for rapid culture: technician (0.70). This finding is consistent with other studies that have shown that SCC-based algorithms have higher SE for detection of Major-IMI than All-IMI (Pantoja et al., 2009, McDougall and Compton, 2014). We hypothesize that this may be due to major-pathogen IMI being more likely than minor pathogen IMI to cause clinical mastitis and marked increases in SCC (Honkanen-Buzalski and Bramley, 1984, Piccart et al., 2016).

Despite the low SE and SP observed in this study, the NPV were moderate to high for All-IMI (0.80) and Major-IMI (0.98). These moderate to high NPV may explain why clinical trials have found that culture- and algorithm-guided SDCT can be implemented without negatively impacting udder health (Bradley et al., 2010, Vasquez et al., 2018,

Rowe et al., In-Press-a, Submitted-b). However, it is important to consider that the high NPV observed in this study are likely to be due to the low prevalence of IMI, as Figure 5.4 shows that the expected NPV declines rapidly at higher prevalences.

In our field study that used the algorithm to make cow-level treatment decisions at dry-off (Rowe et al., Submitted-b), we used a SCC threshold of 200,000 cells/ml. Studies have shown that shifting SCC thresholds induces a near-linear trade-off between SE and SP (Torres et al., 2008, Pantoja et al., 2009, Gohary and McDougall, 2018, Lipkens et al., 2019). Consequently, it is unlikely that a single SCC threshold will perform substantially better than others. Instead, we suggest that SCC thresholds should be selected by producers to match their aspirations for antibiotic reduction and their attitude toward risk for negative impacts on udder health. For example, a producer with a preference towards reducing the risk of infected quarters being omitted from antibiotic therapy (i.e. high SE), should employ a relatively low SCC threshold. The trade-off for this relatively sensitive approach will be a decrease in SP and consequently, a higher proportion of uninfected quarters being treated with antibiotics at dry-off. In contrast, a producer that is relatively motivated to reduce antibiotic use should employ a higher SCC threshold, as this will increase SP and decrease apparent prevalence. However, the disadvantage of a higher SCC threshold is that SE will be lower, resulting in a higher number of infected quarters escaping antibiotic treatment at dry-off, which may negatively affect udder health in the subsequent lactation.

From a practical standpoint, predictive algorithms have significant advantages over other testing strategies. Firstly, algorithms can be executed in advance of the dry-off event, which eliminates the time-lag between the commencement of the dry-off process and the administration of treatments. This is in contrast to other testing strategies evaluated in this study, which requires 4 min (UdderCheck), 45 min (PortaSCC), and 30-40hrs (rapid culture) for the treatment allocation to be determined from the screening test. Secondly, treatment decisions are made at the cow-level, which is considerably simpler than quarter-level treatment, and is therefore more likely to be executed consistently by farm-staff. One of the limitations of algorithm-guided SDCT is that it requires producers to conduct monthly DHIA herd testing and record clinical mastitis events accurately and consistently.

5.5.2 Rapid Culture

The level of agreement between rapid culture and our reference test for prediction of All-IMI status was fair (κ = 0.26-0.28), which was higher than the levels observed for the other tests (κ = 0.01-0.12). For rapid culture: technician, the balance of SE (0.73) and SP (0.63) were such that antibiotic use would be reduced by 55% (as was observed in our clinical trial), while still maintaining a high NPV (0.87). We hypothesize that this high NPV may explain why culture-guided SDCT did not negatively impact udder health in our clinical trial (Rowe et al., In-Press-a, Submitted-b). However, it should be noted that culture- and algorithm-guided SDCT both had very similar reductions in antibiotic use and effects on udder health in our clinical trial, despite the findings from this test validation study indicating numerically better prediction of All-IMI status by rapid culture.

The superior prediction of All-IMI status by rapid culture in this study is unsurprising given that it uses very similar methodology to the reference test, laboratory culture. However there were a number of differences between rapid and laboratory culture in this study. Firstly, rapid culture plates were read by technicians and farm workers, 30-40 hours after incubation, which is in contrast to traditional culture, which were read by experienced microbiologists after 48 hours of incubation. Other differences in testing methodologies included different storage conditions for samples used for rapid culture (chilled for < 6 hrs) and laboratory culture (stored at -20°C for >24hrs) (Murdough et al., 1996), different inoculum volumes for rapid culture (~0.1ml) and laboratory culture (0.01ml) and the use of pathogen-specific thresholds of ≥ 200 cfu/ml for NAS and ≥ 500 cfu/ml for *Bacillus spp* for laboratory culture, but not rapid culture. The substantial agreement ($\kappa = 0.74$) between technicians and farmers in this study indicates that culture-guided SDCT in the hands of farm workers may result in similar reductions and antibiotic use to culture-guided SDCT performed by study technicians.

It is important to consider the logistical requirements for implementing a rapid culture system in a SDCT program. Firstly, rapid culture requires that aseptic milk samples be collected at least two days prior to dry-off, which necessitates substantial allocation of labor and time. Sample collection must be conducted carefully to avoid contamination of milk samples, as it results in bacterial growth, and thus false positives, which ultimately, increases antibiotic use at dry-off. Furthermore, a laboratory must be established either at the farm or at a local veterinary clinic for sample processing, plate incubation, and plate

reading. An additional complicating factor is the use of quarter-level sampling and testing, which adds complexity to the dry-off procedure. Interestingly, we found that rapid culture of composite milk samples had similar SE and SP to quarter-milk samples, indicating that culture-guided SDCT using composite samples could be a simpler alternative to the quarter-sample approach evaluated in our clinical trial (Rowe et al., In-Press-a, Submitted-b). However in this study, the apparent prevalence of All-IMI was slightly higher when testing composite samples (0.56) than quarter samples (0.47), indicating a potential trade-off between simplicity and antibiotic use reduction.

Another important consideration for use of rapid culture in SDCT is the cost of implementation. A partial budget performed in a single-herd study found culture-guided SDCT to be a cost-effective alternative to blanket DCT, but that study did not consider other SDCT approaches (Patel et al., 2017). Consequently, more research is needed to evaluate the economic impact of culture- and algorithm-guided SDCT in U.S. dairy herds. Despite the potential costs and challenges around implementation, we expect that culture-guided SDCT is likely to be an appropriate practice on commercial dairy farms, especially those that do not regularly conduct DHIA herd testing.

5.5.3 Esterase-SCC and Cow-Side LDH

The level of agreement between both PortaSCC and UdderCheck tests and our reference test was poor ($\kappa = 0.01 – 0.12$). Furthermore, both tests tended to strongly favor either SE or SP, and never both, which may impact their utility in the field. For example, PortaSCC ≥ 250 yielded high SE (0.89), but very low SP (0.22) and consequently, a high NPV (0.85) and apparent prevalence (0.81). We expect that using this screening strategy in a

SDCT program would likely result in the majority (85%) of untreated quarters being uninfected, which in theory, may deliver udder health outcomes that are similar to blanket DCT. However, the estimated reduction in antibiotic use would only be 19%, which may not be sufficiently large to justify the cost of implementing the SDCT program. Using the next available cut-point (PortaSCC ≥ 500) resulted in SE, SP, NPV, and apparent prevalence values of 0.40, 0.73, 0.78, and 0.30, respectively. In this situation, we could expect that the antibiotic use at dry-off would be less than with a cut-point of 250 (30% vs 81%), but the proportion of untreated quarters that are uninfected would also decrease from 85% to 78%. It is not clear if a NPV at 0.78 would lead to negative udder health outcomes in the subsequent lactation. However, it should be noted that a NPV of 0.78 is very similar to what would be achieved in a no DCT strategy (i.e. 1 - prevalence = 0.75), and consequently, we would be cautious in implementing such a program. UdderCheck ($\kappa = 0.01$) generally performed worse than PortaSCC ($\kappa = 0.06$ and 0.12), and like PortaSCC, failed to strike a balance between SE/NPV and SP/apparent prevalence. The SE of both tests for detection of Major-IMI was similar to All-IMI, and was slightly worse when composite samples were used. In summary, the performance of esterase tests in this study indicates that their suitability for SDCT is questionable.

The poor performance of PortaSCC ≥ 500 is inconsistent with a recent study that found higher SE for IMI caused by all pathogens at dry-off (SE = 0.78, SP = 0.73), when using the same test and threshold (Kandeel et al., 2019). However, the low SE in this study when using a SCC threshold of 500,000 cells/ml is consistent with previous research that

has found that SE declines when SCC thresholds exceed 200,000 cells/ml (Schepers et al., 1997). To our knowledge, this is the first field study to evaluate an cow-side LDH test. Recent studies using other assays have demonstrated differences in LDH concentrations in milk from quarters with and without IMI (Hernández-Castellano et al., 2017, Khatun et al., 2019). However, direct comparisons between the latter studies and our own are not possible, because those studies did not evaluate LDH as a dichotomous predictor of IMI, and therefore did not report measures of agreement like SE and SP. Other studies have evaluated the ability of LDH at a cut-point of 100 U/L to identify quarters with IMI and concurrently high SCC (Hiss et al., 2007, Kalantari et al., 2013). In those studies, the SE (0.81 and 0.95) and SP (0.86 and 0.94) were higher than observed in our study (SE = 0.76, SP = 0.27), probably due to the inclusion of SCC, which is highly correlated with LDH, in their case definition (Khatun et al., 2019).

From a practical standpoint, these rapid tests could offer some advantages over other screening approaches. Firstly, esterase SCC or cow-side LDH assays do not require that the producer conduct monthly DHIA tests, nor keep records of clinical mastitis cases. Secondly, these tests are faster than rapid culture (<1 hr vs 30-40 hrs), and are likely to have a lower cost per test. The logistical advantages of such testing strategies may be attractive to some producers. However, we recommend that SDCT using these screening tests be evaluated in clinical trials before widespread adoption is considered.

5.5.4 Strengths and Limitations of this Study

Testing samples from cows in commercial herds across multiple regions and laboratories increases the external validity of our study. Furthermore, the pathogen profile observed in our herds was similar to recent surveys of IMI in late lactation cows in the U.S (Rowe et al., 2019a, Rowe et al., In-Press-b), which indicates that tests could perform similarly in the average commercial herd. However, the prevalence of IMI in late lactation cows varies between herds (Rowe et al., 2019a), and it is therefore important to consider how NPV, PPV, and apparent prevalences are likely to change depending on true prevalence (Figure 5.4). Another important strength of this study is the use of multiple tests within the sample population, which allows for quantitative comparisons between tests. The large number of enrolled quarters (~1600) provided a sufficient sample size for precise estimation of test characteristics for prediction of All-IMI status, which is evidenced by relatively narrow 95% confidence intervals. In contrast, only 40 quarters were infected with major pathogens, which is why the estimates for test characteristics were relatively imprecise, as evidenced by wide 95% confidence intervals.

A potential limitation of our study is the imperfect measurement of IMI using laboratory milk culture as the reference test method. Given that SE and SP for laboratory culture are estimated to be approximately 0.65 and 0.95 for All-IMI respectively (Dohoo et al., 2011b), it is possible that the reported test characteristics are biased. This is supported by quantitative bias analysis, which found that SE and SP for rapid culture, algorithm, and PortaSCC® may be underestimated (Table 5.8). Despite the obvious limitations of using an imperfect reference test, we chose to not conduct Bayesian latent class analysis for this study. This was because our study objective was to compare our index tests to traditional

bacteriology, which is the current test of choice for guiding antibiotic therapy. In contrast, a Bayesian latent class model would evaluate the ability of all tests (including the reference test) to predict a 'latent state', that is determined by the model as a function of prior distributions and the pattern of agreement between tests. Even with the use of informative priors for our reference test, we expect that the latent state would be influenced by the other tests, especially given the amount of disagreement between them. Consequently, we chose a traditional frequentist approach, in order to anchor analysis to our reference test and therefore, maintain clarity around our case definition.

5.5.5 Future Research

We plan to conduct economic modelling to evaluate the financial implications of culture- and algorithm-guided SDCT. Future research is needed to better understand the impact of antibiotic treatment among quarters infected with different pathogens to determine if more focused screening protocols can be developed to target IMI that require antibiotic treatment. If proven successful, using a more focused pathogen-based approach to guide strategic treatment decisions could further reduce antibiotic use, thereby making SDCT programs even more attractive and cost-effective to producers. In addition, given the increasing use of technology on farms, we believe there may be an opportunity to evaluate more complex algorithms that include more data points than what has traditionally been used. However, more complex algorithms will only be valuable if they improve upon the diagnostic test characteristics for this method, while still being practical and cost-effective to implement. Finally, given that many algorithms that have been proposed by mastitis experts, research is needed to compare the performance of those algorithms in a single sample of cows in U.S. dairy herds.

5.6 Conclusions

In a cross-sectional study, we compared rapid culture, a predictive algorithm, esterase-SCC, and cow-side LDH tests to traditional culture followed by MALDI-TOF in quarters of cows at dry-off. We found that the kappa values for the level of agreement between the index tests and our reference test ranged from 0.01 (poor) to 0.28 (fair). In general, rapid culture performed better than other tests. The predictive algorithm was slightly more sensitive for Major-IMI than All-IMI. Negative predictive values were moderate to high for all tests, which indicates that implementation of these tests in a SDCT program may result in a small proportion of untreated quarters being infected. Given that an imperfect reference test was used, test characteristics may be underestimated, as indicated by quantitative bias analysis.

5.7 Declaration of competing interest

The Minnesota Easy® 4Cast® plate is manufactured by the University of Minnesota (St. Paul, MN). However, the study investigators have no financial interest in the sale of this plate.

5.8 Authors' roles

Sam Rowe was involved in local and multi-site coordination, fieldwork, laboratory work, data management, statistical analysis and manuscript preparation. Sandra Godden was involved in study conceptualization, herd recruitment in MN and WI, supervision of fieldwork and manuscript editing. Patrick Gorden and Alfonso Lago were involved in

study conceptualization, local site coordination, fieldwork, and manuscript editing. Daryl Nydam was involved in study conceptualization, NY herd recruitment, local site coordination, and manuscript editing. Amy Vasquez was involved in fieldwork, local site coordination, and manuscript editing. Erin Royster was involved in study conceptualization and manuscript editing. Jennifer Timmerman was involved in laboratory work and manuscript editing. Mark Thomas was involved in local site coordination, fieldwork, and manuscript editing.

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Table 5.1: Definition of quarter-level positive and negative results of index tests used to identify infected quarters at dry off.

	Negative / Uninfected	Positive / Infected
Quarter samples		
Rapid culture	No colonies observed	≥1 colonies observed
PortaSCC ≥ 250	<100	250, 500, 750, 1500, >3000
PortaSCC ≥ 500	<100, 250	500, 750, 1500, >3000
UdderCheck ≥ 100	<100	100-200, 200-500, >500
UdderCheck ≥ 200	<100, 100-200	200-500, >500
Composite samples[1]		
Algorithm	Cow met no criteria[2]	Cow met at least 1 of criteria[2]
Rapid culture	No colonies observed	≥1 colonies observed
PortaSCC ≥ 250	<100	250, 500, 750, 1500, >3000
PortaSCC ≥ 500	<100, 250	500, 750, 1500, >3000
UdderCheck ≥ 100	<100	100-200, 200-500, >500
UdderCheck ≥ 200	<100, 100-200	200-500, >500

[1]Quarter-milk samples from a single cow were pooled together into a composite sample either in the lab before testing, or during sampling (i.e. DHIA SCC analysis). If the composite sample tested positive, then all quarters were classified as positive by the index test.
[2]Algorithm criteria: ≥2 cases of CM during lactation, any CM during the 14 d prior to dry-off, or any test day SCC > 200 x 10^3 cells/ml during lactation

Table 5.2: Prevalence of intramammary infection for quarters sampled two days before planned dry-off date

	n	%
Quarters at risk	1424	100.0%
No Growth	1063	74.9%
Infected quarters	361	25.4%
Single	331	23.2%
Mixed	30	2.1%
Major pathogens[1]	40	2.8%
Gram Positive	351	24.6%
Staphylococcus aureus[1]	3	0.2%
Non aureus *Staphylococcus spp.*	232	16.3%
Staphylococcus chromogenes	131	9.2%
Staphylococcus epidermidis	5	0.4%
Staphylococcus haemolyticus	17	1.2%
Staphylococcus hominis	1	0.1%
Staphylococcus sciuri	7	0.5%
Staphylococcus simulans	3	0.2%
Staphylococcus xylosus/saprophyticus	12	0.8%
Staphylococcus sp.	61	4.3%
Streptococcus and Strep-like organisms[1]	32	2.2%
Aerococcus viridans	2	0.1%
Aerococcus sp.	2	0.1%
Enterococcus hirae	1	0.1%
Enterococcus sp.	3	0.2%
Lactococcus garvieae	2	0.1%
Lactococcus sp.	3	0.2%
Streptococcus uberis	2	0.1%
Streptococcus mitis	2	0.1%
Streptococcus sp.	17	1.2%
Other Gram Positive	99	7.0%
Actinomyces sp.	1	0.1%
Bacillus sp.	14	1.0%
Corynebacterium sp.	49	3.4%
Micrococcus luteus	1	0.1%
Micrococcus sp.	21	1.5%
Rothia sp.	2	0.1%
Gram positive coccus	3	0.2%
Gram positive rod	11	0.8%
Gram positive organism	2	0.1%
Gram Negative	11	0.8%
Coliform[1]	4	0.3%
Enterobacter cloacae	1	0.1%
Escherichia coli	2	0.1%
Klebsiella pneumoniae	1	0.1%
Other Gram Negative	7	0.5%
Other organism		
Yeast[1]	2	0.1%

[1]Major pathogens included: *Staphylococcus aureus*, *Streptococcus* and Strep-like organisms, coliforms and yeast

Table 5.3: Frequency of agreement and disagreement for seven index tests compared to the laboratory culture (reference test).

	Number of quarters			
	Ref=1, Index=1	**Ref=1, Index=0**	**Ref=0, Index=1**	**Ref=0, Index=0**
All pathogens				
Algorithm[1,2]	238	123	564	499
Rapid culture: Technician	263	98	396	667
Rapid culture: Farmer	260	101	415	648
PortaSCC ≥ 250	321	40	831	232
PortaSCC ≥ 500	143	218	286	777
UdderCheck ≥ 100	273	88	779	284
UdderCheck ≥ 200	110	251	314	749
Major pathogens[3]				
Algorithm[2]	29	11	773	611
Rapid culture: Technician	28	12	631	753
Rapid culture: Farmer	30	10	645	739
PortaSCC ≥ 250	36	4	1116	268
PortaSCC ≥ 500	16	24	413	971
UdderCheck ≥ 100	31	9	1021	363
UdderCheck ≥ 200	12	28	412	972

[1]Each row of this table is equivalent to a 2 x 2 contingency table.
[2]Algorithm testing was done at the cow-level. All other index tests, and the reference test were conducted at the quarter-level.
[3]Major pathogens included: *Staphylococcus aureus*, *Streptococcus* spp. and Strep-like organisms, coliforms and yeast.

Table 5.4: Test characteristics for six diagnostic approaches using quarter-milk samples for prediction of quarter-level intramammary infection status when considering all pathogens.

	Apparent Prevalence[1]	Sensitivity	Specificity	Youden's index[2]	Positive predictive value	Negative predictive value
Prevalence of IMI = 25%						
Rapid culture: Technician	0.47 (0.44-0.49)	0.73 (0.68-0.77)	0.63 (0.60-0.66)	0.36	0.40 (0.36-0.44)	0.87 (0.85-0.89)
Rapid culture: Farmer	0.48 (0.45-0.50)	0.72 (0.67-0.76)	0.61 (0.58-0.64)	0.33	0.39 (0.35-0.42)	0.87 (0.84-0.89)
PortaSCC ≥ 250	0.81 (0.79-0.83)	0.89 (0.85-0.92)	0.22 (0.19-0.24)	0.11	0.28 (0.25-0.31)	0.85 (0.81-0.89)
PortaSCC ≥ 500	0.30 (0.28-0.32)	0.40 (0.35-0.45)	0.73 (0.70-0.76)	0.13	0.33 (0.29-0.38)	0.78 (0.75-0.81)
UdderCheck ≥ 100	0.74 (0.72-0.76)	0.76 (0.71-0.80)	0.27 (0.24-0.29)	0.02	0.26 (0.23-0.29)	0.76 (0.72-0.80)
UdderCheck ≥ 200	0.30 (0.27-0.32)	0.30 (0.26-0.35)	0.70 (0.68-0.73)	0.01	0.26 (0.22-0.30)	0.75 (0.72-0.77)

[1]The proportion of quarters that test positive to the test of interest. Note that the proportion of quarters classified as infected by the reference test (laboratory culture) was 25%.
[2]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

Table 5.5: Test characteristics for six diagnostic approaches using quarter-milk samples for prediction of quarter-level intramammary infection status when only considering major pathogens[1].

	Apparent Prevalence[2]	Sensitivity	Specificity	Youden's index[3]	Positive predictive value	Negative predictive value
Prevalence of major pathogen IMI = 3%						
Rapid culture: Technician	0.46 (0.44-0.49)	0.70 (0.54-0.82)	0.54 (0.52-0.57)	0.24	0.04 (0.03-0.06)	0.98 (0.97-0.99)
Rapid culture: Farmer	0.47 (0.45-0.50)	0.75 (0.59-0.86)	0.53 (0.51-0.56)	0.28	0.04 (0.03-0.06)	0.99 (0.98-0.99)
PortaSCC ≥ 250	0.81 (0.79-0.83)	0.90 (0.76-0.96)	0.19 (0.17-0.22)	0.09	0.03 (0.02-0.04)	0.99 (0.96-0.99)
PortaSCC ≥ 500	0.30 (0.28-0.33)	0.40 (0.26-0.56)	0.70 (0.68-0.73)	0.10	0.04 (0.02-0.06)	0.98 (0.96-0.98)
UdderCheck ≥ 100	0.74 (0.72-0.76)	0.77 (0.62-0.88)	0.26 (0.24-0.29)	0.04	0.03 (0.02-0.04)	0.98 (0.95-0.99)
UdderCheck ≥ 200	0.30 (0.27-0.32)	0.30 (0.18-0.46)	0.70 (0.68-0.73)	0.00	0.03 (0.02-0.05)	0.97 (0.96-0.98)

[1]Major pathogens included: *Staphylococcus aureus*, *Streptococcus* and Strep-like organisms, coliforms and yeast.
[2]The proportion of quarters that test positive to the test of interest. Note that the proportion of quarters classified as infected by the reference test (laboratory culture) was 3%.
[3]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

Table 5.6: Test characteristics for seven diagnostic approaches using composite milk samples[1] for prediction of quarter-level intramammary infection status when considering all pathogens.

	Apparent Prevalence[2]	Sensitivity	Specificity	Youden's index[3]	Positive predictive value	Negative predictive value
Prevalence of IMI = 25%						
Algorithm	0.56 (0.54-0.59)	0.66 (0.61-0.71)	0.47 (0.44-0.50)	0.13	0.30 (0.27-0.33)	0.80 (0.77-0.83)
Rapid culture: Technician	0.56 (0.53-0.59)	0.76 (0.72-0.81)	0.52 (0.49-0.55)	0.28	0.35 (0.32-0.38)	0.87 (0.84-0.89)
Rapid culture: Farmer	0.52 (0.49-0.55)	0.72 (0.67-0.76)	0.55 (0.52-0.58)	0.27	0.35 (0.32-0.39)	0.85 (0.83-0.88)
PortaSCC ≥ 250	0.88 (0.87-0.90)	0.91 (0.87-0.93)	0.12 (0.10-0.14)	0.03	0.26 (0.24-0.28)	0.79 (0.73-0.85)
PortaSCC ≥ 500	0.37 (0.35-0.40)	0.41 (0.36-0.47)	0.64 (0.61-0.67)	0.05	0.28 (0.24-0.32)	0.76 (0.73-0.79)
UdderCheck ≥ 100	0.78 (0.76-0.80)	0.79 (0.74-0.83)	0.22 (0.20-0.25)	0.01	0.26 (0.23-0.28)	0.75 (0.70-0.80)
UdderCheck ≥ 200	0.31 (0.29-0.34)	0.30 (0.25-0.35)	0.68 (0.65-0.71)	-0.02	0.24 (0.21-0.28)	0.74 (0.71-0.77)

[1]Quarter-milk samples from a single cow were pooled together into a composite sample either in the lab before testing, or during sampling (i.e. DHIA SCC analysis). If a composite sample tested positive, then all quarters were classified as infected by the index test.
[2]The proportion of quarters that test positive to the test of interest. Note that the proportion of quarters classified as infected by the reference test (laboratory culture) was 25%.
[3]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

Table 5.7: Test characteristics for seven diagnostic approaches using composite milk samples[1] for prediction of quarter-level intramammary infection status when considering only major pathogens[2].

	Apparent Prevalence[3]	Sensitivity	Specificity	Youden's index[4]	Positive predictive value	Negative predictive value
Prevalence of major pathogen IMI = 3%						
Algorithm	0.56 (0.54-0.59)	0.72 (0.57-0.84)	0.44 (0.42-0.47)	0.17	0.04 (0.03-0.05)	0.98 (0.97-0.99)
Rapid culture: Technician	0.55 (0.53-0.58)	0.72 (0.57-0.84)	0.45 (0.42-0.48)	0.18	0.04 (0.03-0.05)	0.98 (0.97-0.99)
Rapid culture: Farmer	0.52 (0.49-0.54)	0.75 (0.59-0.86)	0.49 (0.46-0.52)	0.24	0.04 (0.03-0.05)	0.99 (0.97-0.99)
PortaSCC ≥ 250	0.88 (0.87-0.90)	0.92 (0.79-0.98)	0.12 (0.10-0.14)	0.04	0.03 (0.02-0.04)	0.98 (0.95-0.99)
PortaSCC ≥ 500	0.37 (0.35-0.40)	0.42 (0.28-0.58)	0.63 (0.60-0.65)	0.05	0.03 (0.02-0.05)	0.97 (0.96-0.98)
UdderCheck ≥ 100	0.78 (0.76-0.80)	0.80 (0.65-0.90)	0.22 (0.20-0.24)	0.02	0.03 (0.02-0.04)	0.97 (0.95-0.99)
UdderCheck ≥ 200	0.31 (0.29-0.34)	0.30 (0.18-0.46)	0.69 (0.66-0.71)	-0.01	0.03 (0.02-0.05)	0.97 (0.96-0.98)

[1]Quarter-milk samples from a single cow were pooled together into a composite sample either in the lab before testing, or during sampling (i.e. DHIA SCC analysis). If a composite tested positive, then all quarters were classified as infected by the index test.
[2]Major pathogens included: *Staphylococcus aureus*, *Streptococcus* and Strep-like organisms, coliforms and yeast.
[3]The proportion of quarters that test positive to the test of interest. Note that the proportion of quarters classified as infected by the reference test (laboratory culture) was 3%.
[4]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

Table 5.8: Bias-adjusted[1] test characteristics for seven diagnostic approaches for detection of quarter-level intramammary infection status when considering all pathogens.

	Sensitivity	Specificity	Youden's index[2]	Positive predictive value	Negative predictive value
Adjusted prevalence of IMI = 56%					
Composite sample					
Algorithm	0.68	0.50	0.18	0.41	0.75
Quarter-milk sample					
Rapid culture: Technician	0.79	0.71	0.5	0.58	0.87
Rapid culture: Farmer	0.78	0.68	0.46	0.56	0.86
PortaSCC ≥ 250	0.91	0.24	0.15	0.38	0.84
PortaSCC ≥ 500	0.42	0.76	0.18	0.47	0.72
UdderCheck ≥ 100	0.76	0.27	0.03	0.35	0.69
UdderCheck ≥ 200	0.31	0.71	0.01	0.35	0.67

[1]Test characteristics for index tests are calculated, assuming that the reference test has a sensitivity and specificity of 0.8 and 1.0 respectively.
[2]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

Table 5.9: Bias-adjusted[1] test characteristics for seven diagnostic approaches for detection of quarter-level intramammary infection status when considering only major pathogens[1].

	Sensitivity	Specificity	Youden's index[2]	Positive predictive value	Negative predictive value
Adjusted prevalence of IMI = 4%					
Composite sample[3]					
Algorithm	0.72	0.44	0.17	0.05	0.98
Quarter-milk sample					
Rapid culture: Technician	0.70	0.55	0.25	0.05	0.98
Rapid culture: Farmer	0.75	0.54	0.29	0.06	0.98
PortaSCC ≥ 250	0.90	0.19	0.09	0.04	0.98
PortaSCC ≥ 500	0.40	0.70	0.10	0.05	0.97
UdderCheck ≥ 100	0.78	0.26	0.04	0.04	0.97
UdderCheck ≥ 200	0.30	0.70	0.00	0.04	0.96

[1]Test characteristics for index tests are calculated, assuming that the reference test has a sensitivity and specificity of 0.8 and 1.0 respectively.
[1]Major pathogens included: *Staphylococcus aureus*, *Streptococcus* and Strep-like organisms, coliforms and yeast.
[2]Youden's index = Sensitivity + Specificity – 1. Diagnostic tests with equivalent performance as a coin flip have an index value of 0.

$$A = \frac{a - (1 - Specificity) * (a + b)}{Sensitivity + Specificity - 1}$$

$$B = a + b - A$$

$$C = \frac{c - (1 - Specificity) * (c + d)}{Sensitivity + Specificity - 1}$$

$$D = c + d - C$$

Figure 5.1: Formulae used to adjust 2 x 2 contingency tables for the imperfect measurement of intramammary infection. a/A: Ref=1,Index=1, b/B: Ref=0,Index=1, c/C: Ref=1,Index=0,d/D: Ref=0,Index=0. Lowercase letters (a,b,c,d) indicate the observed frequencies. Uppercase letters (A,B,C,D) indicate bias adjusted frequencies.

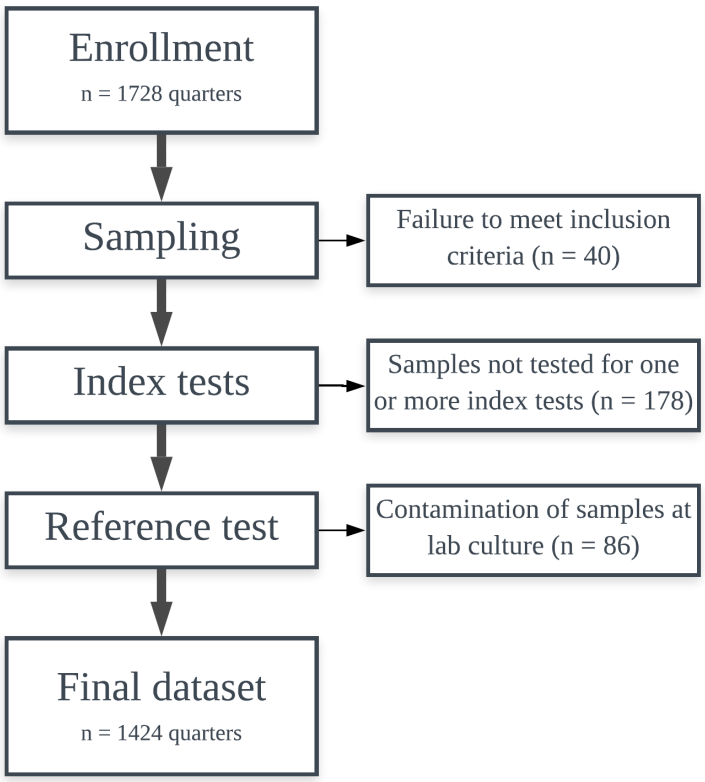

Figure 5.2: Flow of participants (quarters of cows) through the study. Boxes on the right indicate exclusions.

	Laboratory culture	Rapid culture: Technician	Rapid culture: Farmer	Algorithm[1]	PortaSCC ≥ 250	PortaSCC ≥ 500	UdderCheck ≥ 100	UdderCheck ≥ 200
Laboratory culture	1							
Rapid culture: Technician	0.28	1						
Rapid culture: Farmer	0.26	0.73	1					
Algorithm[1]	0.09	0.06	0.04	1				
PortaSCC ≥ 250	0.06	0.04	0.02	0.13	1			
PortaSCC ≥ 500	0.12	0.05	0	0.28	0.18	1		
UdderCheck ≥ 100	0.01	-0.02	-0.04	0.13	0.35	0.21	1	
UdderCheck ≥ 200	0.01	-0.01	-0.03	0.24	0.17	0.53	0.26	1

Figure 5.3: Agreement (Cohen's Kappa) between index and reference tests used in this study. Cells are colored using a gradient from yellow (no agreement) to blue (complete agreement). [1]Algorithm was tested using composite milk samples. All other tests used quarter-milk samples. [for online, not print].

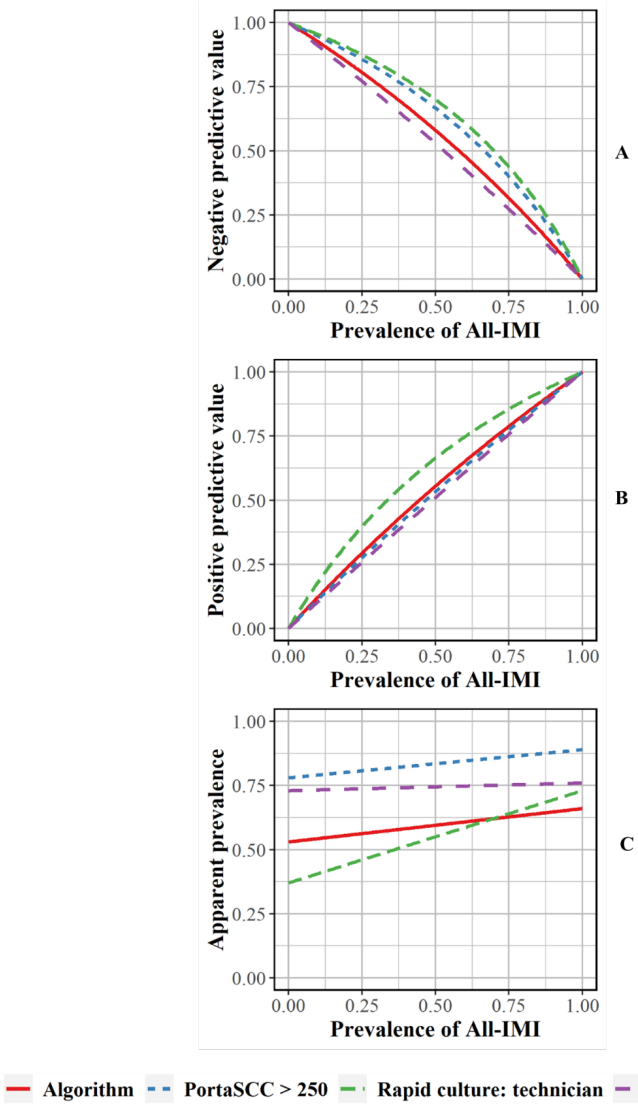

Figure 5.4: Negative predictive values (4A), positive predictive values (4B) and expected apparent prevalences (4C) at different true prevalences of intramammary infection (all pathogens) at dry-off. Apparent prevalence can be interpreted as a proxy for antibiotic use at dry-off, whereas the negative predictive values (NPV) can be interpreted as a proxy for the proportion of untreated quarters being uninfected. Positive predictive values can be used as a proxy for the proportion of treated quarters that are truly infected at dry-off.

6 CHAPTER SIX: Summary of Results, Implications, and Future Directions

6.1 Introduction and Objectives

Mastitis is the most important infectious disease of dairy cows, as it impairs health, production, animal welfare and economic sustainability of the farm. In addition, a large proportion of antibiotic use on dairy farms is for the treatment and prevention of intramammary infections (**IMI**), which includes antibiotic therapy at dry-off (i.e. **DCT**) to cure subclinical IMI that were acquired during lactation and to prevent new IMI during the early dry period. Currently, 80% of U.S. dairy farms practice blanket DCT (**BDCT**), which is a whole-herd approach to DCT (i.e. all quarters are treated). An alternative approach is selective DCT (**SDCT**), which uses a screening test to allocate antibiotic treatments away from cows that are unlikely to benefit from DCT. Recent research in North American herds has found that SDCT can reduce antibiotic use at dry-off by up to 60%. Therefore, SDCT may be a sustainable way for producers to improve antibiotic stewardship. However, there are two major knowledge gaps impeding the widespread implementation of SDCT. Firstly, it is not known what bacteria commonly cause IMI at dry-off, and secondly, no multi-site trials have been conducted in the U.S. to validate SDCT in commercial herds. Consequently, we conducted two field studies to address these specific knowledge gaps. The four studies that were conducted to address our specific aims / objectives are summarized below, followed by a discussion of the implications and future directions in this area.

6.2 Objectives

6.2.1 Objective 1. Describe the Quarter-level Prevalence of Intramammary Infection in Late Lactation Cows in U.S. Dairy Herds

The first objective was to estimate the prevalence of IMI at dry-off in U.S. dairy herds, including a description of the pathogen profile. Such information could be useful for developing SDCT strategies, and informing decisions around antibiotic therapy at dry-off (eg. drug choice). Eighty herds were recruited in a multi-site cross-sectional study. Herds were purposively selected to achieve near-equal representation of four bedding materials of interest: manure solids, organic non-manure, new sand and recycled sand bedding. Each herd was visited twice for sampling (summer 2017 and winter 2017-18) and aseptic quarter-milk samples were collected (n = 10,448) from late lactation cows. We found that quarter-level prevalence of IMI was 21.1%, which was primarily caused by non-aureus *Staphylococcus* spp. (**NAS**, 11.4%) and *Streptococcus* and Strep-like organisms (**SSLO**, 5.6%). Prevalence of coliforms was very low (0.15%). Findings from this study indicate that quarter-level IMI prevalence in late lactation cows is low in this study population of herds, which suggests that SDCT could result in a more efficient use of antibiotics than BDCT in some U.S. herds. Furthermore, the low prevalence of coliforms indicates that dry cow antibiotics with Gram-negative spectrum of activity may not be necessary on many farms.

6.2.2 Objective 2: Describe Associations between Bedding Type, Bedding Bacteria Count and Intramammary Infection Risk in Late Lactation Cows

For the second objective we aimed to evaluate if bedding type and/or bedding bacteria counts were associated with IMI prevalence in quarters of late lactation cows. Preventing the development of IMI during lactation could reduce the proportion of quarters that are

infected and therefore require antibiotic treatment at dry-off. This was the same 80-herd cross-sectional study described for Objective 1. In addition to collecting aseptic quarter milk samples, we also collected unused bedding samples and used bedding samples from the late lactation cow pens. Aerobic culture was used to enumerate counts in bedding (log_{10} CFU / ml) of all bacteria, *Staphylococcus spp., Streptococcus spp*. and Strep-like organisms (SSLO), Coliforms, *Klebsiella spp*, non-coliform Gram-negatives*, Bacillus spp*. and *Prototheca spp*. Generalized linear mixed models (family = binomial, link = logit) were performed to evaluate the relationship between herd-level bedding bacteria counts (log10 CFU/ml) and quarter-level IMI risk. Herd-level bedding type was also evaluated as a predictor of quarter-level IMI risk using the same methods. In general, the association between bacteria counts in unused bedding and IMI was positive (odds ratio [**OR**] = 1.08, 95% CI: 1.00 – 1.17), which was mostly consistent across all four common bedding material types. In contrast, the association between counts of all bacteria in used bedding and IMI varied by bedding type, with positive associations observed in quarters exposed to manure solids (OR = 2.29, 95% CI: 1.15 – 4.54) and organic non-manure (OR = 1.51, 95% CI: 1.09 – 2.09) and a negative association in quarters exposed to new sand (OR = 0.47, 95% CI: 0.26 – 0.87). Only modest differences in IMI prevalence were observed between the four common bedding material types. In conclusion, our findings indicate that bedding may function as a reservoir for pathogens that cause IMI that are detected at dry-off. Further research using prospective, experimental designs could help to confirm hypotheses generated from this cross-sectional, observational study.

6.2.3 Objective 3. Determine the Effect of Selective Dry Cow Therapy on Udder Health

The third objective was to evaluate culture- and algorithm- guided SDCT in a multi-site trial in U.S. herds. Cows (n=1275) were enrolled from seven herds from five U.S. states (MN, WI, IA, CA, NY) and randomized to BDCT (control group), culture- and algorithm-guided SDCT at dry-off. At dry-off, BDCT cows received an intramammary antibiotic (500mg ceftiofur hydrochloride) in all four quarters. Antibiotic treatments were selectively allocated to quarters of culture-guided SDCT cows by only treating quarters from which aseptically collected milk samples tested positive on the Minnesota Easy® 4Cast® plate (University of Minnesota. St. Paul, MN) after 30-40 hours of incubation. For algorithm-guided SDCT cows, antibiotic treatments were selectively allocated at the cow-level, with all quarters receiving antibiotic treatment if the cow met at least one of the following criteria: 1) any dairy herd improvement association (**DHIA**) test with a SCC > 200,000 cells / ml during the current lactation; 2) two or more clinical mastitis cases during the current lactation; and/or 3) one or more clinical mastitis cases in the 14 days before enrollment. All quarters of all cows were treated with an internal teat sealant (**ITS**).

Outcomes of interest included dry period IMI dynamics, rates of clinical mastitis and removal from the herd (culling or death) during 1-120 days in milk (**DIM**), and average daily SCC and milk yield during 1-120 DIM. We found that both SDCT programs resulted in a 55% reduction in antibiotic use, as compared to BDCT, although the reduction in antibiotic use varied between individual study herds. Additionally, all health outcomes were similar between treatment groups, with effect estimates and confidence

intervals being close to zero (absolute measures like risk differences) and one (relative measures like hazard ratios). The findings indicate that under the conditions of this trial, SDCT can have negligible effects on udder health while significantly reducing antibiotic use at dry-off.

6.2.4 Objective 4. Describe the Characteristics of Screening Tests Commonly Used in Selective Dry Cow Therapy

It is possible that different tests could be used successfully for the purpose of identifying infected cows or quarters in SDCT programs. As such, our fourth objective was to compare four tests to standard milk culture followed by MALDI-TOF in quarters of cows at dry-off. The four index tests included rapid milk culture, a predictive algorithm, an esterase strip test measuring SCC and a cow-side lactate dehydrogenase (**LDH**) test. Cows (n=432) that were randomly allocated to the culture-guided SDCT group in our multi-site trial (Objective 3) were included in this cross-sectional study. Aseptic foremilk samples were collected from quarters (n=1728) two days prior to dry-off, and subjected to index and reference tests. Rapid culture was performed using the Minnesota Easy® 4Cast® plate. For the algorithm test method, all quarters were classified as infected if the cow met any of the following criteria: 1) any DHIA test with a SCC > 200,000 cells / ml during the current lactation; 2) two or more clinical mastitis cases during the current lactation; or 3) one clinical mastitis case in the 14 days prior to enrollment. Esterase-SCC and cow-side LDH tests involved adding milk to the test strip and reading for color changes. The reference test was traditional aerobic culture conducted in an accredited laboratory using MALDI-TOF for identification of isolates.

Unconditional logistic regression was used to estimate sensitivity, specificity, apparent prevalence, positive predictive value, negative predictive value for each test. Cohen's Kappa (κ) was used to measure agreement between tests. Algorithm, esterase-SCC and cow-side LDH tests had poor agreement with the reference test (κ ranging from 0.01 to 0.12), while rapid culture had fair agreement (κ = 0.28). No test had concurrently high (>0.8) sensitivity and specificity. Negative predictive values were moderate to high for all tests, which may explain why culture- and algorithm-guided SDCT had negligible impacts on udder health in the clinical trial (Objective 3).

6.3 Implications and Opportunities for Future Research

The overarching aim of these studies was to generate evidence to inform dry cow therapy practices in the U.S. Our 80-herd cross-sectional study found that the quarter-level prevalence of IMI in U.S. herds was approximately 21%, indicating that BDCT is unlikely to be an efficient use of antibiotics on many U.S. farms (i.e. for every 1 infected quarter that is treated under BDCT, another 4 uninfected quarters are also treated unnecessarily). Improving bedding cleanliness during the lactation period may be one important strategy to further reduce the prevalence of IMI, and therefore antibiotic use, at dry-off. Our multi-site clinical trial showed that both culture- and algorithm guided-SDCT could be used to significantly reduce antibiotic use at dry-off (55% reduction) in commercial herds without negatively impacting future udder health or performance. These findings indicate that SDCT can be used to improve antibiotic stewardship on farms.

6.3.1 Next Steps for Bedding Research

Cross-sectional studies similar to our 80-herd study could be conducted using cows from earlier stages of lactation. This would allow for additional insights into the relationship between bedding hygiene and the development of mastitis during lactation. A small number of observational studies (including this one) have demonstrated an association between BBC and IMI. However, no studies have shown that reducing BBC improves udder health. Therefore, intervention studies could be conducted to evaluate management practices hypothesized to improve udder health. Furthermore, we plan to use the dataset from this study to investigate the relationship between bedding management practices, bedding characteristics (eg. moisture and organic matter concentrations) and bedding bacteria counts.

6.3.2 Further Improvement of Selective Dry Cow Therapy Programs

We plan to conduct economic modelling to evaluate the financial implications of culture- and algorithm-guided SDCT. Future research is needed to better understand the impact of antibiotic treatment among quarters infected with different pathogens to determine if more focused screening protocols can be developed to target IMI that require antibiotic treatment. If proven successful, using a more focused pathogen-based approach to guide strategic treatment decisions could further reduce antibiotic use, thereby making SDCT programs even more attractive and cost-effective to producers. In addition, given the increasing use of technology on farms, we believe there may be an opportunity to evaluate more complex algorithms that include more data points than have traditionally been used. This could include the use of regression or machine-learning methods for generating predictive models. However, more complex algorithms will only be valuable

if they improve upon the diagnostic test characteristics for this method, while still being practical and cost-effective to implement. Finally, given that many algorithms have been proposed by mastitis experts, research is needed to compare the performance of those algorithms in a single sample of cows in U.S. dairy herds.

6.3.3 Re-Evaluating the Importance of Dry Cow Therapy

This thesis has focused on SDCT as an alternative to BDCT. We did not evaluate dry-off practices using no antibiotics, such as an ITS-only approach. This approach would have significantly less logistical constraints than SDCT as no screening test is needed. However, mastitis experts are reluctant to recommend a no-DCT approach, citing decades of research showing superior udder health outcomes in cows receiving DCT, when compared to negative controls. However, to our knowledge, no negatively controlled trials have been conducted to evaluate the efficacy of DCT in U.S. dairy herds in the past 20 years. During that time, the profile of pathogens causing IMI during the dry period has changed significantly and the use of ITS to prevent new infections over the dry period has become increasingly popular. Therefore, we hypothesize that the proportion of cows likely to benefit from DCT in well-managed herds that use ITS has diminished over time, such that DCT may no longer be necessary in those herds. Re-evaluation of the need for DCT in negatively controlled trials is relevant and timely. If ITS-only is shown to be inferior to BDCT and/or SDCT, then such studies will generate contemporary evidence to caution producers about the risks of practicing ITS-only programs. Such evidence is currently needed as an increasing number of herds are suspending DCT in response to challenging economic conditions within the industry.

Furthermore, if ITS-only is found to have equivalent health outcomes to BDCT, then we expect that there will be further research to evaluate ITS-only approaches.

7 CHAPTER SEVEN: REFERENCES

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