The effect of intrauterine administration of mannose or bacteriophage on uterine health and fertility of dairy cows with special focus on *Escherichia coli* and *Arcanobacterium pyogenes*

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ABSTRACT

The objective of this study was to evaluate the effects of intrauterine administration of 50 g of ultrapure mannose or a bacteriophage cocktail and the presence of *Escherichia coli* and *Arcanobacterium pyogenes* in the uterine lumen on uterine health and reproductive performance of lactating dairy cows. The study was conducted on a commercial dairy farm located near Ithaca, New York, from May 4 to January 20, 2011, and 597 cows were enrolled. The cows were divided randomly into 3 treatment groups, and treatments were administered at 2 ± 1 d in milk (DIM). Treatment 1 consisted of intrauterine administration of 50 g of ultrapure mannose powder divided in 4 pills; treatment 2 consisted of intrauterine administration of a bacteriophage cocktail that included 4 different phages in one pill, with a dose of approximately 10^7 plaque-forming units; and treatment 3 consisted of intrauterine administration of one empty pill (control). Intrauterine fluid swabs were collected on day of treatment and at 10 ± 3 DIM; uterine lavage samples were collected at 35 ± 3 DIM. Swabs and uterine lavage samples were cultured for *E. coli* and *A. pyogenes*. The intrauterine administration of mannose and bacteriophage did not affect uterine health, reproduction performance, or outcome of intrauterine secretion cultures for *E. coli* and *A. pyogenes*. Prevalence of intrauterine *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 2 ± 1 , 10 ± 3 , and 35 ± 3 were affected by retained placenta. Additionally, prevalence of intrauterine *E. coli* at 10 ± 3 and *A. pyogenes* at 35 ± 3 DIM were associated with metritis, and cows that were diagnosed with clinical endometritis at 35 ± 3 DIM had greater prevalence of intrauterine *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 35 ± 3 DIM. Furthermore, cows positive for *E. coli* at 2 ± 1 and 10 ± 3 DIM and for *A. pyogenes* at 10 ± 3 DIM had 1.63, 2.34, and 1.54

increased odds of having metritis. Cows positive for *A. pyogenes* at 35 ± 3 DIM and for *E. coli* at 2 ± 1 DIM had 19.8 and 2.66 higher odds of being diagnosed with clinical endometritis, respectively. Additionally, cows negative for *E. coli* at 2 ± 1 DIM were 1.39 times more likely to conceive than cows positive cows for *E. coli*.

Key words: dairy cow, uterine cultures, bacteriophage, mannose

INTRODUCTION

Postpartum uterine diseases (specifically puerperal metritis, clinical endometritis, and subclinical endometritis) are important for animal welfare and economic reasons because they contribute to cow discomfort, elimination from the herd, and impaired reproductive performance. Puerperal metritis is defined as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge associated with signs of systemic illness (decreased milk yield, dullness, or other signs of toxemia) and temperature $>39.5^\circ\text{C}$ within 21 d after parturition, whereas endometritis is the inflammation of the uterus without systemic illness (Sheldon et al., 2006).

The uterine lumen is usually contaminated by bacteria after parturition (Foldi et al., 2006). The composition of the bacterial flora in the postpartum uterus of dairy cows differs between healthy cows and cows with metritis (Santos et al., 2011). *Escherichia coli*, *Arcanobacterium pyogenes*, and *Fusobacterium necrophorum* are considered important etiological agents of uterine diseases (Miller et al., 2007; Bicalho et al., 2010a; Santos et al., 2011). Moreover, the *E. coli* *fimH* gene is prevalent and expressed in *E. coli* that infect the postpartum uterus (Bicalho et al., 2010a; Sheldon et al., 2010). The FimH protein is an *E. coli* type 1 pili adhesive protein that plays an important role in the adhesion to mannoses (Krogfelt et al., 1990) and enables bacteria to colonize epithelial surfaces (Mooi and de Graaf, 1985). It is known that *E. coli* expressing the type 1 pili containing FimH causes urinary tract infection in humans (Kaper et al., 2004), and it is critical

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for the ability of these *E. coli* to adhere to and colonize the bladder epithelium (Mulvey, 2002). Additionally, the presence of the *fimH* gene in intrauterine *E. coli* plays an important role in postpartum uterine diseases (Bicalho et al., 2010a). Immunization with FimH prevents *E. coli* colonization of the bladder mucosa in mice (Langermann et al., 1997), and oral treatment with FimH antagonists has the potential to reduce *E. coli* colonization in the bladder and could be an effective treatment for urinary tract infection in humans (Klein et al., 2010). Sheldon et al. (2010) reported that adhesion of intrauterine *E. coli* to endothelial cells is at least partly mediated by FimH. Therefore, mannose (a FimH antagonist) could bind to intrauterine *E. coli* FimH, preventing *E. coli* from adhering to the uterine mucosa (Sheldon et al., 2010) and potentially reduce the subsequent presence of *A. pyogenes* in the uterine lumen (Dohmen et al., 2000), thus reducing the occurrence of uterine diseases (Bonnett et al., 1991; Bondurant, 1999). This assumption is partly supported by the fact that King et al. (1998) reported that mannose might be effective in reducing bacterial infection in the equine endometrium.

Bacteriophages are viruses that infect bacteria; they are obligate intracellular parasites because they lack their own metabolism. Phages are host specific, able to infect only specific species or even strains of bacteria (Barrow and Soothill, 1997). Optimistic results regarding their use against certain bacterial infections have been revealed by recent randomized clinical trials in mice and cattle. Mice were successfully rescued with a single injection of a phage solution containing 3×10^8 pfu of a certain bacteriophage after inoculated with lethal doses of vancomycin-resistant *Enterococcus faecium* (Biswas et al., 2002). Additionally, Smith and Huggins (1983) reported that bacteriophage therapy was successful when newborn, colostrum-deprived calves were inoculated with *E. coli* and treated with intramuscular inoculation with bacteriophages. Moreover, our research group isolated environmental bacteriophages using *E. coli* isolated from postpartum uterus (Bicalho et al., 2010b) and evaluated the in vitro antimicrobial activity of a bacteriophage cocktail against intrauterine *E. coli* (Santos et al., 2010). This cocktail was able to inhibit in vitro growth of *E. coli* isolates but its in vivo effectiveness is yet to be tested.

Ideally, treatments for uterine diseases should control pathogens from the uterus and should result in withdrawal periods for milk and meat that are as short as possible (Azawi, 2008). Systemic or intrauterine antibiotic therapy is commonly used as the treatment for metritis (Azawi, 2008). However, extensive use of antimicrobials in food animal production has contributed to the emergence of antimicrobial resistance among

pathogens, which complicates the treatment of infectious diseases (Tollefson et al., 1999).

The presence of *E. coli* and *A. pyogenes* in the uterus is associated with uterine inflammation and impaired reproductive performance (Studer and Morrow, 1978; Bonnett and Martin, 1995; Bondurant, 1999). Although Studer and Morrow (1978) reported that the uterine presence of both cultures was associated with poor reproductive performance, Bonnett and Martin (1995) and Williams et al. (2005) found that only the presence of *A. pyogenes* in the uterus was associated with reproductive failure.

Therefore, the objective of this study was to evaluate the effectiveness of 2 alternative treatments (50 g of ultrapure mannose or a phage cocktail that was effective in killing intrauterine *E. coli* in vitro) for preventing metritis and reducing uterine contamination. Additionally, this study aimed to investigate the association of uterine presence of *A. pyogenes* and *E. coli* at 3 time points (2 ± 1 , 10 ± 3 , and 35 ± 3 DIM) with uterine health and reproduction performance.

MATERIALS AND METHODS

Farm and Management

The study was conducted on a commercial dairy farm located near Ithaca, New York, from May 4, 2010, to January 20, 2011. This farm was selected because of its long working relationship with the Ambulatory and Production Medicine Clinic at Cornell University. The farm milked 2,800 Holstein cows 3 times daily in a double 52-stall parallel milking parlor. The cows were housed in freestall barns with concrete stalls covered with mattresses and bedded with manure solids. All cows were offered a TMR consisting of approximately 55% forage (corn silage, haylage, and wheat straw) and 45% concentrate (corn meal, soybean meal, canola, cottonseed, and citrus pulp) on a DM basis. The diet was formulated to meet or exceed the National Research Council nutrient requirements (NRC, 2001) for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM. Reproductive management used a combination of Presynch (Moreira et al., 2001), Ovsynch (Pursley et al., 1995), Resynch (Fricke et al., 2003), and detection of estrus, with 25 to 30% of cows bred via timed AI and the remainder bred after detection of estrus solely by activity monitors (Alpro, DeLaval, Kansas City, MO).

Treatment Groups and Case Definition

Before the study, statistical power and sample size calculations were performed. Considering a sample size

of 200 cows per group, a statistical power of 0.8, a *P*-value of 0.05, and an overall incidence of metritis of 20%, a reduction in the metritis incidence of 50% or more between treatment groups would have been statistically significant. In total, 597 cows were enrolled in the study and there were no study exclusion criteria. A total randomized field trial study design was used; cows were randomly allocated into 1 of 3 treatments groups using the random number function of Excel (Microsoft Corp., Redmond, MA). Treatment 1 consisted of intrauterine administration of 50 g of ultrapure mannose powder divided into 4 pills; 50 g of mannose is 10 times the amount used by King et al. (1998), when they effectively reduced bacterial infection in the equine endometrium. The cow's uterus is enlarged after parturition; therefore, we proposed to increase the mannose dose for this study. Treatment 2 consisted of intrauterine administration of a bacteriophage cocktail that included 4 phages in one pill with titration of approximately 10^7 pfu. The bacteriophages used in the cocktail were isolated and tested in vitro in previous studies (Bicalho et al., 2010b; Santos et al., 2010); multiplicity of infection (MOI) as low as 10^{-4} affected the growth of *E. coli* when tested in vitro. Because the quantity of *E. coli* present in the postpartum uterus was unknown, the bacteriophage treatment dose was selected based on treatment production limitations. Treatment 3 consisted of the intrauterine administration of one empty pill, which was considered the control treatment. The pills used for the treatment were made from porcine gelatin (Torpac, Fairfield NJ). The treatments were administered at 2 ± 1 DIM by the research team as follows: cows were restrained and the perineum area was cleansed and disinfected with 70% ethanol solution. Then, a sterile swab (Har-Vet McCullough Double-Guarded Uterine Culture Swab, Spring Valley, WI) covered by a sterile pipette (inside a plastic sheath) was introduced to the cranial vagina. The pipette was manipulated through the cervix into the uterus. There, the sheath was ruptured and the swab was exposed to uterine secretion. The swab was pulled inside the pipette and kept in transportation medium at 4°C until it was processed in the laboratory. Subsequently, the pills were introduced with a clean sleeve to the cranial vagina and then manually through the cervix into the uterus. Swabs were cultured aerobically on MacConkey agar (Difco, Becton Dickinson, Franklin Lakes, NJ) at 37°C, and *E. coli* colonies were distinguished by a purple-red color. The swabs were also cultured on Mueller-Hinton agar plates (BBL, Becton Dickinson) supplemented with 5% defibrinated sheep blood for 48 h aerobically in 5% CO₂ at 38°C. Typical *A. pyogenes* colonies were distinguished by

colony morphology, postincubation hemolysis, and characteristic appearance on Gram stain. Metritis diagnosis was performed at 10 ± 3 DIM by the research team as follows: the uterus was palpated to exteriorize uterine fluids, and the presence of fetid, watery, red-brown uterine discharge was defined as metritis (Sheldon et al., 2006). Metritis peaks in the first 7 d after calving (LeBlanc, 2008); however, because of study logistics, we could only diagnose metritis at 10 ± 3 DIM. A swab of the uterine fluids was taken and cultured as described above.

Clinical endometritis was traditionally diagnosed primarily by presence of a mucopurulent vaginal discharge (LeBlanc et al., 2002; Sheldon et al., 2006). More recently, it has been established that many cows with purulent or mucopurulent vaginal discharge are free of endometrial inflammation (Dubuc et al., 2010a,b). We therefore adapted our diagnostic procedure. Clinical endometritis was evaluated at 35 ± 3 DIM by visual inspection of a uterine lavage sample obtained as described by Gilbert et al. (2005). In this way, we were able to ensure that visible signs of inflammation (purulent or mucopurulent exudate) emanated from the uterus, rather than from another site. Uterine lavage sampling was performed as follows: the cow was restrained, the perineum area was cleansed and disinfected with 70% ethanol, and a plastic infusion pipette was introduced into the cranial vagina and manipulated through the cervix into the uterus. A total of 20 mL of sterile saline solution was infused into the uterus and agitated gently, and a sample of the fluid was aspirated. The volume of recovered fluid ranged from 5 to 15 mL. All of the samples were visually scored by one investigator, who assessed the presence of a purulent or mucopurulent secretion in the uterine lavage sample. The score ranged from 0 to 2, with 0 indicating absence of a purulent or mucopurulent secretion in the lavage sample, 1 indicating a bloody but not purulent sample, and 2 the presence of pus in the lavage sample. Cows with a score of 2 were considered as diagnosed with clinical endometritis. Samples were kept in ice until they were processed in the laboratory and cultured as described above.

Body condition scores were recorded at the time of each sampling using a 5-point scale with a quarter-point system as described by Edmonson et al. (1989). Stillbirth was defined as death of a calf occurring just before, during, or within 48 h of parturition (Philipsen et al., 1979). Retained placenta was defined as a condition where cows fail to release their fetal membranes within 24 h of calving (Kelton et al., 1998), and it was diagnosed by the farm workers. Data regarding reproductive performance were extracted from the

farm's DairyComp 305 database (Valley Agricultural Software, Tulare, CA).

Statistical Analysis

To facilitate data analysis and interpretation, the variables BCS loss (**LOSS**, 1 if the cow lost BCS from 1 ± 2 to 35 ± 3 DIM; 0 if cow maintained or gained BCS from 1 ± 2 to 35 ± 3 DIM), presence of clinical endometritis at 35 ± 3 (**ENDOM**, yes = 1 and no = 0), presence of metritis 10 ± 3 (**MET**, yes = 1 and no = 0), presence of retained placenta at 2 ± 1 (**RP**, yes = 1 and no = 0), the outcome of the uterine lavage sample culture for *E. coli* at 2 ± 1 , 10 ± 3 and 35 ± 3 DIM (**ECOLI1**, yes = 1 and no = 0; **ECOLI2**, yes = 1 and no = 0; **ECOLI3**, yes = 1 and no = 0), and the outcome of the uterine lavage sample culture for *A. pyogenes* at 2 ± 1 , 10 ± 3 , and 35 ± 3 DIM (**APYO1**, yes = 1 and no = 0; **APYO2**, yes = 1 and no = 0; **APYO3**, yes = 1 and no = 0) were created. Additionally, descriptive statistics analysis was undertaken in SAS using the FREQ procedure (SAS Institute Inc., Cary, NC).

Two logistic regression models were fitted in SAS using the Logistic procedure to examine in detail the association of the independent variables treatment (mannose = 1, phage = 2, and control = 3), parity, **ECOLI1**, **ECOLI2**, **APYO1**, **APYO2**, **APYO3**, **LOSS**, and **RP** with the odds of metritis and endometritis incidence. The association between the different uterine culture outcomes in different time points was evaluated with the use of Chi-squared analysis in JMP 9 (SAS Institute Inc., Cary, NC).

The effect of several independent variables on reproduction was analyzed by Cox's proportional hazard using the proportional hazard regression procedure in SAS. For this analysis of reproduction, cows were right-censored if not diagnosed as being pregnant before culling, death, or the end of the data collection period, which was around 210 DIM. Variables offered to the models included treatment (mannose = 1, phage = 2, and control = 3), parity, **MET**, **ENDOM**, **ECOLI1**, **ECOLI2**, **APYO1**, **APYO3**, **LOSS**, and **RP**. To illustrate the effect of **ECOLI1** on reproduction, Kaplan-Meier survival analysis was performed using MedCalc version 11.5.1.0 software (MedCalc Software, Mariakerke, Belgium); the Logrank test was used to compute *P*-values.

For all models described above, independent variables and their respective interactions were kept when $P < 0.10$ in an attempt to reduce the type II error risk while maintaining a stringent type I error risk of 5%. The variable treatment was forced into all statistical models, even in the absence of statistical significance.

RESULTS

The 2 treatments tested did not have any effect on the results of the outcome of intrauterine secretions cultures (Table 1), were unsuccessful in the prevention of metritis or endometritis (Table 3), and did not affect reproductive performance (Table 5). However, we forced the variable treatment into all statistical models.

The treatments did not affect the proportion of cows bred via timed AI. The percentage of cows bred via timed AI was 22.25, 25.33, and 27.83% for cows in the mannose, bacteriophage, and control groups, respectively ($P = 0.14$).

The descriptive statistics of the effect of risk factors and results regarding the outcome of the intrauterine secretion cultures are described in Table 1. Briefly, parity and BCS did not affect any of the outcomes of intrauterine secretion cultures. Retained placenta was the most important risk factor for the presence of *E. coli* and *A. pyogenes* in intrauterine secretions. Prevalence of intrauterine *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 2 ± 1 , 10 ± 3 , and 35 ± 3 DIM were affected by retained placenta. Additionally, prevalence of intrauterine *E. coli* at 10 ± 3 and *A. pyogenes* at 35 ± 3 DIM were associated with metritis. Moreover, cows that were diagnosed with clinical endometritis at 35 ± 3 DIM had greater prevalence of intrauterine *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 35 ± 3 DIM.

The association between intrauterine culture outcomes at different time points is described in Table 2. In brief, the prevalence of *E. coli* at 10 ± 3 DIM and *A. pyogenes* at 2 ± 1 DIM were significantly associated with presence of *E. coli* at 2 ± 1 DIM. Moreover, the presence of *E. coli* at 10 ± 3 DIM was negatively associated with the prevalence of *A. pyogenes* at this same time point. Additionally, the presence of *A. pyogenes* at 2 ± 1 DIM was significantly associated with the prevalence of *A. pyogenes* at 35 ± 3 DIM.

Parity and presence of *E. coli* at 2 ± 1 and 10 ± 3 DIM and *A. pyogenes* at 10 ± 3 in intrauterine secretions were significantly associated with the odds of metritis (Table 3). Cows in first and second lactation had 3.8 and 1.78 higher odds of being diagnosed with metritis compared with cows in third or greater parity, respectively ($P < 0.001$). Moreover, cows positive for *E. coli* at 2 ± 1 and 10 ± 3 DIM and for *A. pyogenes* at 10 ± 3 DIM had 1.63, 2.34, and 1.54 increased odds of having metritis, respectively. Presence of *E. coli* at 2 ± 1 DIM and of *A. pyogenes* at 35 ± 3 DIM were significantly important for the odds of endometritis (Table 4). Cows that were positive for *A. pyogenes* at 35 ± 3 DIM had 19.8 higher odds of being diagnosed with clinical endometritis ($P < 0.001$). Additionally, cows that were *E. coli* positive at 2 ± 1 DIM had

Table 1. Descriptive statistics of the effect of risk factors on the outcome of the intrauterine secretion cultures

Risk factor	Cows, no.	Intrauterine <i>E. coli</i> , % (95% CI)			Intrauterine <i>A. pyogenes</i> , % (95% CI)		
		2 ± 1 DIM	10 ± 3 DIM	35 ± 3 DIM	2 ± 1 DIM	10 ± 3 DIM	35 ± 3 DIM
Treatment ¹							
Mannose	188	44.9 (37–53) ^a	64.8 (56–73) ^a	9.3 (5–15) ^a	39.2 (32–47) ^a	45.8 (38–54) ^a	12.8 (8–19) ^a
Phage	218	35.7 (29–43) ^a	66.5 (59–74) ^a	8.4 (5–13) ^a	34.2 (27–41) ^a	39.7 (32–48) ^a	16.3 (11–21) ^a
Control	191	42.2 (35–50) ^a	72.6 (65–78) ^a	10.1 (6–15) ^a	28.3 (22–36) ^a	47.3 (39–56) ^a	13.5 (9–19) ^a
Lactation							
Parity 1	264	37.5 (31–44) ^a	68.7 (62–75) ^a	9.1 (6–13) ^a	34.6 (29–41) ^a	47.1 (40–54) ^a	13.0 (9–18) ^a
Parity 2	171	41.2 (33–50) ^a	63.9 (55–72) ^a	10.1 (6–16) ^a	34.5 (27–43) ^a	41.0 (32–50) ^a	13.9 (9–20) ^a
Parity ≥3	162	45.7 (37–55) ^a	71 (61–79) ^a	8.5 (5–14) ^a	31.8 (24–41) ^a	41 (31–51) ^a	17.0 (11–24) ^a
RP ²							
No	560	37.7 (33–42) ^a	66.6 (62–71) ^a	9.8 (7–13) ^a	32.2 (28–36) ^a	45.8 (41–51) ^a	13.2 (10–16) ^a
Yes	37	89.7 (73–98) ^b	86.7 (69–96) ^a	0 ^a	62.1 (42–79) ^b	20 (8–39) ^b	32.3 (17–51) ^b
Metritis ³							
No	385	37.5 (32–43) ^a	61.5 (55–67) ^a	10.2 (7–13) ^a	31.0 (26–36) ^a	41.4 (35–47) ^a	10.8 (8–14) ^a
Yes	196	45.5 (38–53) ^a	77.7 (71–84) ^b	7.2 (4–12) ^a	39.5 (32–47) ^a	48.6 (41–56) ^a	21.5 (16–28) ^b
Endometritis ⁴							
No	472	37.6 (33–42) ^a	67.2 (62–72) ^a	8.0 (6–11) ^a	32.1 (28–37) ^a	46.3 (41–52) ^a	6.3 (4–9) ^a
Yes	97	55.9 (45–67) ^b	71.2 (60–81) ^a	15.0 (8–24) ^a	42.9 (32–54) ^a	33.7 (23–45) ^a	53.8 (43–64) ^b
BCS ⁵							
>3	49	31.3 (24–39) ^a	67.1 (59–65) ^a	10.0 (6–15) ^a	33.7 (26–41) ^a	47.3 (39–56) ^a	10.6 (6–16) ^a
3	50	43.5 (37–50) ^a	67.9 (61–74) ^a	10.0 (7–14) ^a	32.1 (26–38) ^a	42.4 (36–49) ^a	16.2 (12–21) ^a
<3	481	48.9 (38–60) ^a	69.6 (58–79) ^a	5.8 (2–12) ^a	39.1 (29–50) ^a	43.0 (32–55) ^a	15.4 (9–24) ^a

^{a,b}Values with different superscripts within the same column and within a risk factor differ ($P < 0.05$).

¹Mannose treatment consisted of the intrauterine administration of 50 g of ultrapure mannose powder divided in 4 pills; phage treatment consisted of the intrauterine administration of a bacteriophage cocktail in one pill with titration of approximately 10^7 pfu; control consisted of intrauterine administration of one empty pill. Treatments were performed at 2 ± 1 DIM.

²RP = retained placenta, where cows fail to release their fetal membranes within 24 h of calving.

³Metritis = presence of fetid, watery, red-brown uterine discharge at 10 ± 3 DIM.

⁴Endometritis = presence of pus in the uterine lavage sample performed at 35 ± 3 DIM.

⁵BCS at 10 ± 3 DIM, using a 5-point scale with a quarter-point system as described by Edmonson et al. (1989).

Table 2. Association of intrauterine culture outcomes in different time points

Culture outcome	Cows, no.	<i>Escherichia coli</i> positive, %			<i>Arcanobacterium pyogenes</i> positive, %		
		2 ± 1 DIM	10 ± 3 DIM	35 ± 3 DIM	2 ± 1 DIM	10 ± 3 DIM	35 ± 3 DIM
<i>E. coli</i> culture							
At 2 ± 1 DIM							
Negative	322	—	63 ^a	9 ^a	31 ^a	45 ^a	13 ^a
Positive	236	—	77 ^b	8 ^a	39 ^b	43 ^a	16 ^a
At 10 ± 3 DIM							
Negative	149	29 ^a	—	5 ^a	30 ^a	55 ^a	16 ^a
Positive	330	44 ^b	—	9 ^a	33 ^a	39 ^b	16 ^a
At 35 ± 3 DIM							
Negative	517	41 ^a	67 ^a	—	33 ^a	45 ^a	14 ^a
Positive	51	40 ^a	79 ^a	—	41 ^a	35 ^a	16 ^a
<i>A. pyogenes</i> culture							
At 2 ± 1 DIM							
Negative	366	38 ^a	67 ^a	8 ^a	—	46 ^a	12 ^a
Positive	192	46 ^b	71 ^a	11 ^a	—	41 ^a	20 ^b
At 10 ± 3 DIM							
Negative	270	40 ^a	74 ^a	9 ^a	34 ^a	—	18 ^a
Positive	209	39 ^a	60 ^b	6 ^a	30 ^a	—	13 ^a
At 35 ± 3 DIM							
Negative	487	40 ^a	68 ^a	9 ^a	32 ^a	46 ^a	—
Positive	80 ^a	46 ^b	68 ^a	10 ^a	46 ^b	36 ^a	—

^{a,b}Values with different superscripts within the same column differ ($P < 0.05$).

Table 3. Effects of treatment, lactation number, and the outcome of the intrauterine secretion cultures (for *Escherichia coli* and *Arcanobacterium pyogenes*) on the odds of metritis incidence estimated with the use of logistic regression models

Variable	Cows, no.	Metritis ² incidence, %	Adjusted odds ratio	P-value
Mannose ¹	188	35.5	0.87	0.86
Phage	218	30.5	0.93	
Control	191	35.0	Baseline	
Parity 1	264	47.8	3.80	<0.001
Parity 2	171	25.0	1.78	
Parity ≥ 3	162	17.4	Baseline	
<i>E. coli</i> negative (2 ± 1 DIM)	322	30.0	Baseline	0.02
<i>E. coli</i> positive (2 ± 1 DIM)	236	37.4	1.63	
<i>E. coli</i> negative (10 ± 3 DIM)	149	27.1	Baseline	<0.001
<i>E. coli</i> positive (10 ± 3 DIM)	330	44.7	2.34	
<i>A. pyogenes</i> negative (10 ± 3 DIM)	270	36.0	Baseline	
<i>A. pyogenes</i> positive (10 ± 3 DIM)	209	42.9	1.54	0.05

¹Mannose treatment consisted of the intrauterine administration of 50 g of ultrapure mannose powder divided in 4 pills; phage treatment consisted of the intrauterine administration of a bacteriophage cocktail in one pill with titration of approximately 10^7 pfu; control consisted of intrauterine administration of one empty pill. Treatments were performed at 2 ± 1 DIM.

²Metritis = presence of fetid, watery, red-brown uterine discharge at 10 ± 3 DIM.

2.66 higher odds of having clinical endometritis ($P = 0.002$).

A multivariable Cox proportional hazard model was performed to evaluate the effect of several variables on reproduction (Table 5). The only variables retained in this model were treatment, parity, and the presence of *E. coli* in intrauterine secretions at 2 ± 1 DIM. Treatment had no effect on reproduction. Conversely, cows in parity 1 and 2 were 1.6 and 1.5 more likely to conceive than cows in parity ≥ 3 , respectively ($P = 0.02$). Cows that were negative for *E. coli* at 2 ± 1 DIM were 1.39 times more likely to conceive than cows positive for *E. coli*. By 200 DIM, 80% of cows those negative for *E. coli* at 2 ± 1 DIM were pregnant, and only 68% of positive cows had conceived (Figure 1).

DISCUSSION

We were encouraged by results of previous studies to perform this clinical trial with the aim of establishing alternatives for prophylaxis and treatment for post-partum uterine diseases. Given the growing interest in reducing the use of antibiotics in farm animals, any positive results from this study could be considered important.

Bacteriophages could be beneficial for metritis treatment because decreasing the bacterial load of *E. coli* is likely to decrease the severity of the disease (Sheldon et al., 2006). Bacteriophages have been successfully used in animal trials against a broad range of pathogenic *E. coli* (Brüssow, 2005). Previous stud-

Table 4. Effects of treatment, lactation number, and the outcome of the intrauterine secretion cultures (for *Escherichia coli* and *Arcanobacterium pyogenes*) on the odds of endometritis incidence estimated with the use of logistic regression models

Variable	Cows, no.	Endometritis ¹ incidence, %	Adjusted odds ratio	P-value
Mannose ²	188	15.1	0.65	0.55
Phage	218	17.7	0.93	
Control	191	17.3	Baseline	
Parity 1	264	19.0	1.22	0.09
Parity 2	171	11.3	0.50	
Parity ≥ 3	162	19.0	Baseline	
<i>E. coli</i> negative (2 ± 1 DIM)	322	12.0	Baseline	0.002
<i>E. coli</i> positive (2 ± 1 DIM)	236	22.4	2.66	
<i>A. pyogenes</i> negative (35 ± 3 DIM)	487	9.1	Baseline	<0.001
<i>A. pyogenes</i> positive (35 ± 3 DIM)	80	63.3	19.8	

¹Endometritis = presence of pus in the uterine lavage sample performed at 35 ± 3 DIM.

²Mannose treatment consisted of the intrauterine administration of 50 g of ultrapure mannose powder divided in 4 pills; phage treatment consisted of the intrauterine administration of a bacteriophage cocktail in one pill with titration of approximately 10^7 pfu; control consisted of intrauterine administration of one empty pill. Treatments were performed at 2 ± 1 DIM.

Table 5. Cox's proportional hazard analysis evaluating the effect of treatment¹ (mannose, phage, and control) on the hazard of pregnancy; the variables parity and first *Escherichia coli* culture (DIM = 2 ± 1) were also retained in the model

Variable	Cows, no.	Adjusted hazard ratio	P-value
Mannose	188	0.92	0.89
Phage	218	0.65	
Control	191	Baseline	
Parity 1	264	1.6	0.02
Parity 2	171	1.5	
Parity ≥ 3	162	Baseline	
<i>E. coli</i> negative (2 ± 1 DIM)	322	Baseline	0.008
<i>E. coli</i> positive (2 ± 1 DIM)	236	0.72	

¹Mannose treatment consisted of the intrauterine administration of 50 g of ultrapure mannose powder divided in 4 pills; phage treatment consisted of the intrauterine administration of a bacteriophage cocktail in one pill with titration of approximately 10^7 pfu; control consisted of intrauterine administration of one empty pill. Treatments were performed at 2 ± 1 DIM.

ies isolated environmental bacteriophages using *E. coli* isolated from postpartum uterus, estimated their lytic activity (Bicalho et al., 2010b), and evaluated the in vitro antimicrobial activity of a bacteriophage cocktail against intrauterine *E. coli* (Santos et al., 2010). This cocktail had a notable capacity to inhibit the in vitro growth of *E. coli* isolates. Unfortunately, in the current study, bacteriophage therapy did not have any effect

on metritis incidence. A possible explanation could be that bacteriophages are host specific, able to infect only specific species or even strains of bacteria (Barrow and Soothill, 1997). In vitro, MOI = 10^1 and MOI $>10^2$ were efficient in inhibiting 50 and 90% of all isolates tested, respectively (Santos et al., 2010). Even though the bacteriophage cocktail showed good effectiveness in killing intrauterine *E. coli* in vitro, the genetic vari-

Figure 1. Kaplan-Meier survival analysis of calving-to-conception interval by the outcome of intrauterine secretion culture of *Escherichia coli* (DIM = 2 ± 1). Cows that were positive for intrauterine *E. coli* culture at 2 ± 1 DIM (dotted line) had a median calving-to-conception interval of 142 d compared with 101 d for cows that were negative for *E. coli* (solid line).

ability of intrauterine *E. coli* strains is large (Bicalho et al., 2010a), and the quantity of *E. coli* present in the postpartum uterus was unknown. This might have affected the spectrum of activity of the bacteriophages used and could have decreased the effectiveness of the cocktail in vivo. Additionally, the activity of the phages when in the intrauterine environment is unknown, and the lochia secretion present in early postpartum uterus might have inhibited bacteriophage activity. However, a previous study showed that in a model system that simulated intestinal conditions, an *Enterobacter aerogenes*-specific bacteriophage persisted for a prolonged period and proliferated in reactors simulating the different parts of the large intestine (Verthé et al., 2004). Furthermore, Waseh et al. (2010) reported that salmonella colonization in chickens was reduced by orally administered bacteriophage. These studies show that bacteriophages can survive and maintain their activity under in vivo conditions; however, further research is needed in this area.

The inclusion of the mannose treatment was also encouraged by previous studies. Bicalho et al. (2010a) found that FimH, an *E. coli* type 1 pili adhesive protein that plays an important role in adhesion to mannoses and enables the bacteria to colonize epithelial surfaces (Mooi and de Graaf, 1985), is very important for metritis and endometritis. In addition, King et al. (1998) reported that mares receiving a 100-mL intrauterine infusion of *E. coli* (10^9 cells suspended in sterile PBS) alone accumulated more intrauterine fluid at 24 h than mares that received 100 mL of *E. coli* (10^9 cells) suspended in PBS containing 50 mg of mannose/mL, whereas prevalence of positive culture at 24 h was significantly reduced in the mannose-treated group. Ultrapure mannose was used because it would be applied as an intrauterine treatment; hence, it would be in direct contact with intrauterine *E. coli*. Mannose treatment in vitro was efficient in decreasing the ability of intrauterine *E. coli* to adhere to endometrial cells (Sheldon et al., 2010). One strong explanation for the ineffectiveness of this treatment in the current study is that the mannose concentration was unlikely to be at the same level (2.5%) as used in the in vitro test (Sheldon et al., 2010). In early postpartum, the uterus is enlarged and the uterine volume is probably greater than 2 L, which was the exact uterine volume needed to have an intrauterine mannose concentration of 2.5%. However, the quantity of mannose used in this study was 10 times more than was used in a previous trial (King et al., 1998). Other possibilities could decrease the mannose concentration in the uterus; for example, part of the treatment could have been carried out of the uterus by lochia; osmotic pressure could have been caused by the introduction of the treatment in the uterine lumen, and

this might have caused a decrease in mannose concentration in the uterus; or the mannose could have been used as a carbon source by bacteria (Kornberg and Lambourne, 1992). Additionally, the uterine environment might influence the binding mechanism between mannose and FimH.

In total, 597 cows were enrolled on this trial; the observed metritis incidence was 35.5, 30.5, and 35% and that of endometritis was 15.1, 17.7, and 17.3% for the mannose, phage, and control groups, respectively. Therefore, given our sample size and the observed differences in uterine disease incidence, we estimated that our study had a statistical power of 0.7 to 0.8 to demonstrate that the evaluated treatments had no effect on the incidence of metritis and endometritis.

Although the treatments tested were not effective for prevention of uterine diseases, interesting findings regarding uterine bacteriology in 3 periods are shown in this study. Retained placenta was the most important risk factor for uterine contamination, principally in the first 3 DIM. Before calving, the uterus is sterile, whereas after parturition, bacteria may enter the uterine lumen ascending from the environment (Foldi et al., 2006); retained placenta is an important risk factor for uterine bacterial contamination (Paisley et al., 1986) and is also known to be an important risk factor for metritis (Bruun et al., 2002). Additionally, in the present study, first- and second-lactation cows were more likely to be diagnosed with metritis. This can be explained by the fact that the incidence of dystocia is higher in cows in first and second lactation (Bonneville-Hébert et al., 2011), and dystocia is one of the risk factors of metritis (Bruun et al., 2002; Benzaquen et al., 2007; Ghavi Hossein-Zadeh and Ardalan, 2011). However, first- and second-parity cows had better reproductive performance, and this result is supported by previous studies (Benzaquen et al., 2007; Machado et al., 2010).

Presence of *E. coli* at 2 ± 1 DIM had the most detrimental effect on uterine health and reproductive performance. Presence of *E. coli* at 2 ± 1 and 10 ± 3 DIM and of *A. pyogenes* at 10 ± 3 DIM were significantly important for the odds of metritis incidence. These results are supported by previous studies that found that *E. coli* and *A. pyogenes* were two of the most prevalent bacterial species associated with uterine diseases and reproductive failure (Studer and Morrow, 1978; Bonnett et al., 1991; Bondurant, 1999). Additionally, a study performed in buffalos reported that *E. coli* and *A. pyogenes* were mainly present in uteri affected by metritis (Azawi et al., 2007). Moreover, presence of *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 35 ± 3 DIM were important for the odds of endometritis incidence; however, only the presence of *E. coli* at 2 ± 1 DIM impaired reproductive performance. It has been

reported that uterine *E. coli* are merely opportunistic environmental bacteria, because none of the virulence factors evaluated were associated with the probability of occurrence of uterine diseases (Silva et al., 2009). On the other hand, Bicalho et al. (2010a) reported that *E. coli* virulence factors, such as *fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA*, and *astA*, were associated with uterine diseases and impaired reproductive performance, and these 5 virulence factors were not evaluated in the Silva et al. (2009) study. Additionally, Dohmen et al. (2000) reported that the presence of *E. coli* and intrauterine endotoxin in the first 2 DIM were positively associated with the presence of *A. pyogenes* plus gram-negative bacteria at 14 DIM, supporting the hypothesis that an early postpartum uterine *E. coli* infection would affect uterine health. However, this same association was not observed in this study; the presence of *E. coli* at 2 ± 1 DIM was not directly associated with the presence of *A. pyogenes* at 10 ± 3 DIM. Presence of *E. coli* at 2 ± 1 DIM was associated with subsequent presence of *E. coli* at 10 ± 3 DIM, which was also associated with the incidence of metritis. This supports our findings that the presence of *E. coli* at 2 ± 1 DIM was important for uterine health parameters and impaired reproduction performance. However, Williams et al. (2005) did not find any association between *E. coli* and reproduction failure, and reported that the only species associated with impaired reproduction performance was *A. pyogenes*. Furthermore, Bonnett and Martin (1995) found that the presence of *A. pyogenes* at 26 DIM was associated with poor reproductive performance.

CONCLUSIONS

In conclusion, intrauterine administrations of 50 g of mannose or bacteriophage cocktail at 2 ± 1 DIM were not effective in improving uterine health in dairy cows. Additionally, we found that parity and presence of *E. coli* at 2 ± 1 and 10 ± 3 DIM and that of *A. pyogenes* at 10 ± 3 DIM in intrauterine secretions were significantly associated with the odds of metritis. Furthermore, presence of *E. coli* at 2 ± 1 DIM and *A. pyogenes* at 35 ± 3 DIM were significantly important for the odds of endometritis. Additionally, cows negative for *E. coli* at 2 ± 1 DIM were more likely to conceive than cows positive for *E. coli* at this time point.

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