Short communication: Effect of 3 phytoceutical products on elimination of bacteria in experimentally induced *Streptococcus uberis* clinical mastitis

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ABSTRACT

Our objective was to assess the ability of 3 herbal products to eliminate experimentally induced *Streptococcus uberis* mastitis. These herbal products, also known as phytoceuticals, are used in organically managed dairy cattle to maintain or promote udder health. The products tested were an intramammary product, a topical product, and a product applied to the vulvar area. These products are not approved by the US Food and Drug Administration for treatment of mastitis but they are sold to enhance milk quality or for maintenance or improvement of udder health. Each of the products contains at least one component shown to have antibacterial activity. In this study, we successfully challenge-inoculated 25 lactating dairy cows maintained under organic conditions with an isolate of *S. uberis*. All challenged cows were positive for *S. uberis* by milk culture after challenge. When cows met predefined criteria indicating the presence of clinical mastitis, treatment with 1 of the 3 products was initiated based upon a predetermined random allocation. Culture of aseptically collected quarter milk samples was performed before, during, and following challenge with *S. uberis*. Eight, 8, and 9 cows received the intravulvar, intramammary, and topical treatments, respectively. Milk from all cows that were treated with phytoceuticals were culture-positive for *S. uberis* at every time point following treatment through 168 h following the last phytoceutical treatment. Based upon the presence of clinical signs and for humane reasons, 2 intravulvar-treated cows, 1 topical-treated, and 4 intramammary-treated cows received intramammary antibiotic therapy. We concluded that the phytoceuticals tested, as dosed and used in this trial, did not produce bacterial cures in *S. uberis*-induced mastitis.

Key words: mastitis, *Streptococcus uberis*, phytoceutical

Short Communication

Plant-based products (phytoceuticals) that are not approved by the US Food and Drug Administration are marketed to dairy farmers to improve milk quality and udder health. In some cases, these products are used by organic dairy producers for treatment of mastitis. A wide range of products, from mint-based topical udder salves to essential oil blends, are marketed for intramammary use, with limited scientific evidence of their efficacy or knowledge of the duration that indicator molecules are present in milk or tissues. Our research group examined 3 phytoceutical products in an attempt to determine the potential for residues from key antibacterial molecules present in the products when used in lactating dairy cattle (Armorini et al., 2016). We studied pharmacokinetics in healthy dairy cattle, as well as in dairy cattle with experimentally induced clinical mastitis (Mason et al., 2017, 2018; Mullen et al., 2017). In addition to pharmacokinetic and residue data, our research allowed us to investigate the influence of these products on the course of experimentally induced *Streptococcus uberis* clinical mastitis. In that respect, the hypothesis tested was that these phytoceuticals would be effective in producing bacteriological cure of experimentally induced clinical bacterial mastitis.

We tested 3 phytoceutical products that are marketed to dairy producers. Dr. Paul's CEG Tincture (Dr. Paul's Lab, Arcadia, WI) contains extracts of cayenne, *Echinacea*, and garlic in apple cider vinegar and was administered intravulvar at 3 mL once daily for 3 d, as recommended by the manufacturer (IVU treatment). Phyto-Mast (CowMaster LLC, Narvon, PA) contains thyme essential oil and extracts of several other plants (*Angelica* spp., *Gaultheria* sp., and *Glycyrrhiza* sp.). The recommended dose of this product is two 12.0-mL tubes inserted into the affected quarter via the teat canal (intramammary) once every 12 h for 2 doses total (IMM treatment). Uddersol (Ralco Animal Health, Marshall, MN) is a proprietary blend of oregano essential oil and olive oil. The recommended dosage is 25 mL applied topically to the affected udder quarter (TOP treat-

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ment). Each of these 3 products contains at least one ingredient shown to have antibacterial activity in vitro, including garlic (Cowan, 1999), thyme (Mullen et al., 2014b), and oregano (Ananda Baskaran et al., 2009). Because of this potential for antimicrobial activity, in addition to the pharmacokinetic studies, we collected quarter milk samples aseptically for microbiological culture before and after intramammary challenge with *S. uberis*.

Thirty mid- to late-lactation multiparous Holstein, Jersey, or Holstein cross cows were purchased from certified organic dairies in North Carolina, Virginia, and West Virginia. To qualify for the study, cows were required to have composite quarter sample SCC <400,000/mL and no growth in the quarter to be inoculated on at least 2 occasions, as well as either no growth or infection with minor pathogen species only (CNS or *Corynebacterium* spp.) in the remaining quarters. The average ($\pm$ SD) composite quarter milk sample SCC for all cows selected was $58,000 \pm 70,000$ cells/mL (range 4,000 to 318,000 cells/mL, with only 2 cows >200,000 cells/mL). Three cows had one quarter infected with CNS, and 3 other cows had one quarter infected with *Corynebacterium* spp. at the time of screening. These 6 cows had composite SCC <55,000 cells/mL, and the quarter that was culture-positive at purchase was not used for the experimental induction of mastitis.

Cows were housed in the Laboratory Animal Resources facilities at the North Carolina State University College of Veterinary Medicine under Institutional Animal Care and Use protocol 14-086-A. Average daily milk production for the cows based upon the 3 milkings before inoculation was 11.6 ± 0.6 kg/d (mean $\pm$ SD). All cows were housed in the same indoor temperature-controlled facility, fed a certified organic concentrate and free choice certified organic alfalfa-orchardgrass hay, and were milked twice daily by machine. Only 10 cows could be housed at a time in the facilities, so phytoceutical treatments administered to cows were randomized among the groups of cows over time.

Culture-negative status in the study quarter was confirmed by 2 milk samples collected 24 h apart upon the cow's arrival at the research facility. Qualifying cows were challenge-inoculated with *S. uberis* strain 0140J in a single rear quarter (except for 1 cow that was inoculated in the left front quarter) at 2 to 4 h after milking. The *S. uberis* strain, obtained from J. Hogan (The Ohio State University), was originally isolated from bovine clinical mastitis (Finch et al., 1997). Preparation of the inocula was as published by Kester et al. (2015), with some modifications. Inocula of *S. uberis* were prepared by adding 2 cfu of an 18- to 24-h solid medium culture of *S. uberis* to 10 mL of tryptic

soy broth (Hardy Diagnostics, Santa Maria, CA). Vials were incubated for 18 h in a 37°C shaking water bath at 100 rpm. Following incubation, the broth was aliquotted into microfuge tubes and centrifuged at $6,600 \times g$ for 15 min at 4°C. Pellets were reconstituted with sterile PBS (Hardy Diagnostics) and serially diluted. As described by Kester et al. (2015), the percent transmission at 540 nm of each dilution was measured (ThermoScientific Genesys, Madison, WI) and the dilution closest to 71% transmission was adjusted to exactly 71% using sterile PBS. Serial dilutions were used in an attempt to achieve a target inoculum concentration of 2,300 cfu/mL of PBS for intramammary infusion. A sterile teat cannula was fitted to a 3-mL syringe containing 1 mL of inoculum. On the day of the challenge inoculations, inocula were cultured for enumeration (cfu/mL) of *S. uberis*. Although initial inocula were low and estimated at ≤ 30 cfu/mL, one cow (#12) challenged with this dose became infected, with repeated positive post-challenge milk cultures. The procedure was adjusted thereafter and inocula contained approximately 1,000 to 4,000 cfu in 1.0 mL. Challenge doses used in various studies have varied with the challenge strain, cattle age, lactation stage, and other trial factors. Challenge doses have ranged from 250 cfu (Paape et al., 1988) to 10^6 cfu (Notcovich et al., 2016). Responses have also varied based upon the criteria used for selection of experimental animals. Two studies (Khazandi et al., 2015; Notcovich et al., 2016) have shown large variations in response to different strains of *S. uberis* isolates obtained from clinical mastitis.

Challenge inoculation was performed 2 to 4 h after milking. The selected rear teat (in one exception, a front teat was used) was aseptically prepared for inoculation by dipping in the teat in 1% iodine teat dip (Dairy Star EconoStar 10K, GEA Farm Technologies Inc., Bönen, Germany), wiping the teat using a paper towel, and then scrubbing the teat end with 70% ethanol-soaked cotton balls. The inoculum was administered into the quarter using partial insertion of the teat cannula, and the quarter was massaged for 5s to distribute the inoculum.

Cows were evaluated at every milking (twice daily) by scoring attitude, appetite, milk appearance, and udder pain, size, and temperature (Table 1). Cows were considered eligible to be dosed with phytoceuticals when clinical mastitis was detected, based upon the following criteria for the challenged quarter and cow: A milk score ≥ 3 (see Table 1) for at least one milking and one or more of the following: fever (temperature above 39.4°C; 103°F); swelling of the mammary gland (increased more than 33% of its normal size); heat in the affected quarter (palpably warmer than other

Table 1. Scoring scales for cow and mammary gland assessments¹

Scale	Attitude	Appetite	Milk	Udder pain	Udder size	Udder temperature
1	Normal	Normal	Normal	None	None	Normal
2	Slight depression	Slight decrease	Small flakes	Slight	Slight increase (10–33%)	Slight warmth
3	Moderate depression	Moderate decrease	Clots	Moderate	Moderate increase (34–66%)	Moderate warmth
4	Marked depression	Marked decrease	Serous (with or without clots)	Marked	Marked increase (67–100%)	Marked warmth
5	Severe depression	Severe anorexia	Hemorrhagic	Severe	Decreased	Cold

¹Modified from that described by Anderson et al. (1986).

quarters and warmer than temperature of abdominal surface skin); and obvious pain in the mammary gland (strongly reacting or kicking when palpated).

Dosing with phytoceuticals was performed as described. The IMM treatment was administered following disinfection of the teat end using 70% ethanol-soaked cotton balls. The udder quarter was massaged for 3 s after administration of each tube and for an additional 5 s after administration of both tubes. The IVU treatment was administered by insertion of the 3-mL syringe 5 cm into the vagina at an upward angle followed by slow release of the product into the vagina. The TOP treatment was administered by placing the product into a gloved hand and rubbing on the skin over the entire lateral skin surface of the challenged udder quarter, from the intermammary groove continuing dorsally to the attachment of the udder to the body at the line of reflection from udder to thigh, and to the mid-point of both quarters on the lateral side.

Milk samples for microbiological culture from the inoculated quarter were collected before inoculation, 12, 24, and 48 h after inoculation, at dosing with phytoceutical, and at the following times after the last dose of the phytoceutical: 12, 24, 48, 72, 96, and 168 h (7 d). Cows still showing clinical signs of mastitis (milk or gland abnormalities with signs of systemic illness; Score 3 of AABP Clinical Mastitis Severity Scoring System; AABP, 2016) after phytoceutical administration were administered intramammary antibiotic therapy (ceftiofur, Spectramast LC, Zoetis, Parsippany, NJ) as shown in Table 2.

One of the 30 cows was found to be infected in the challenged quarter with an organism other than *S. uberis*, and this cow was eliminated from the study. An additional 4 cows did not become culture-positive, even after repeated challenges, and data on those cows are not presented. The results of intramammary challenge of the remaining 25 cows are shown in Table 2.

A minimum of 3 d was allotted to determine whether the challenge inoculation was successful. If the challenge inoculation was determined to be unsuccessful, then the cow was reinoculated. Previous research on induced clinical mastitis using this strain of *S. uberis* in lactating dairy cattle indicated that all cattle exhibited clinical signs of mastitis within 4 d of challenge (Kester et al., 2015), at an average time of 48 h but up to 4 d (de Greeff et al., 2013), and in as little as 18 to 24 h (Moyes et al., 2009). In these prior studies, selection of cows was based upon criteria more stringent than those used here and the cows appeared to have been obtained from a single research herd. This may, at least partially, explain the disparate responses observed between our study and prior studies.

A total of 8 infected cows received IVU, 8 infected cows received IMM, and 9 infected cows received TOP. All cows treated with phytoceuticals were culture-positive for *S. uberis* at every time point following treatment through 168 h following the last phytoceutical treatment, except in cases where cows received intramammary antibiotic therapy. Based on the presence of clinical signs of mastitis and for humane reasons, 2 IVU cows, 1 TOP cow, and 4 IMM cows received intramammary antibiotic therapy (Spectramast LC). Of these 7 cows, 4 were treated twice (2 IMM, 1 IVU, and 1 TOP) and 3 were treated 3 times (2 IMM and 1 IVU). Although there were limited post-antibiotic treatment cultures, milk cultures from available affected quarters were culture-negative for those cows from which post-treatment cultures were obtained.

Previous research examined the efficacy of IMM (Phyto-Mast) for treatment of naturally occurring clinical mastitis and reported that the product was ineffective for resolution of mastitis with no significant reduction in SCS from IMM treatment (Pinedo et al., 2013). Another study examined the use of IMM as a dry-off therapy and found no adverse effects as

Table 2. Results of intramammary challenge with *Streptococcus uberis* and treatment with phytoceuticals

Cow no.	Quarter challenged ¹ (SCC ×10 ³ /mL at challenge)	No. of total challenges	Challenge inoculum (cfu/mL)	Result of challenges ²	Product administered ³	Outcome	Treated with ceftiofur
1	LR, 64	3	≤30, ≤30, 1,000	Success	IVU	Still infected	Yes
2	LR, 4	2	≤30, ≤30	Success	IVU	Still infected	No
3	LR, 68	3	≤30, ≤30, 1,000	Success	IVU	Still infected	No
4	RR, 45	3	≤30, ≤30, 1,000	Success	IMM	Still infected	No
5	LR, 43	3	≤30, ≤30, 1,000	Success	IMM	Still infected	No
6	RR, 14	2	≤30, ≤30	Success	TOP	Still infected	No
7	RR, 110	3	≤30, ≤30, 1000	Success	TOP	Still infected	No
8	LR, 122	2	≤30, ≤30	Success	TOP	Still infected	No
9	RR, 114	2	≤30, ≤30	Success	TOP	Still infected	Yes
10	RR, 82	2	≤30, 1,700	Success	IVU	Still infected	No
11	RR, 272	3	≤30, 1,700, 1,950	Success	TOP	Still infected	No
12	LR, 72	1	≤30	Success	IVU	Still infected	No
13	RR, 41	2	≤30, 1,700	Success	IMM	Still infected	No
14	RR, 166	2	≤30, 1,700	Success	IMM	Still infected	Yes
15	LR, 188	2	≤30, 1,700	Success	IMM	Still infected	Yes
16	LR, 138	3	≤30, 1,700, 1,950	Success	TOP	Still infected	No
17	LR, 532	2	≤30, 1,700	Success	TOP	Still infected	No
18	LR, 86	2	≤30, 1,700	Success	TOP	Still infected	No
19	LR, 237	2	1,600, 3,180	Success	IVU	Still infected	No
20	LR, 38	1	1,600	Success	TOP	Still infected	No
21	LR, 54	1	1,600	Success	IVU	Still infected	Yes
22	RR, 41	1	1,600	Success	IMM	Still infected	Yes
23	LR, 10	2	1,600, 3,180	Success	IMM	Still infected	No
24	LF, ⁴ 409	1	1,600	Success	IMM	Still infected	Yes
25	RR, 44	1	1,600	Success	IVU	Still infected	No

¹LR = left rear; RR = right rear; LF = left front. Somatic cell count at challenge differed from that described in text, which was at qualification.

²Success indicates presence of clinical signs and milk from the challenged quarter being culture-positive for *S. uberis*.

³IVU = Dr. Paul's CEG Tincture (Dr. Paul's Lab, Arcadia, WI), with extracts of cayenne, *Echinacea*, and garlic in apple cider vinegar administered intravulvar; IMM = Phyto-Mast (CowMaster LLC, Narvon, PA), with thyme essential oil and extracts of several other plants (*Angelica* spp., *Gaultheria* sp., and *Glycyrrhiza* sp.) administered intramammary; TOP = Uddersol (Ralco Animal Health, Marshall, MN), proprietary blend of oregano essential oil and olive oil, administered topically.

⁴Cow 24 was the exception to the case that rear quarters were always challenged; cow 24 had minor infections in both rear quarters and was inoculated in the LF quarter.

measured by milk production and SCC (Mullen et al., 2014a). From these data, phytoceuticals have not been found to adversely affect milk production or increase SCC, but the tested phytoceuticals also do not appear to effectively treat mastitis as measured by SCC.

One limitation of this study was that only one dose and treatment duration of each phytoceutical was tested. However, we followed the manufacturers' recommendations and believed that having a larger number of cows treated with one dose and duration was preferable to using variations in dose and duration in a smaller number of cows. Another limitation was the initial difficulty we encountered in quantifying our challenge inoculum. However, all cows counted as successful inoculations did show recovery in milk of the presumed challenge strain of *S. uberis*, and cows were not treated until they showed signs of clinical mastitis. Because of the lack of strain typing of the isolates, the introduction of a "wild" strain from the environment or fecal contamination is possible but unlikely.

We recognize that the current study was not designed with the primary purpose of an efficacy study for mastitis therapy. The phytoceutical products tested contain compounds with in vitro antibacterial activity. Because we only tested one dose, we cannot speculate on the response to different or higher phytoceutical doses, nor whether the tested products might have greater efficacy against other mastitis-causing microorganisms. Based on our results, we can state that the 3 products tested as described here did not eliminate clinical *S. uberis* introduced by intramammary challenge.

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