Efficacy of *Panax ginseng* extract combined with cephalexin as a dry cow therapy

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SUPPLEMENTARY FILE

Materials & Methods

Panax ginseng extract (PGe)

A 50 mg/mL PGe stock solution was prepared by dissolving the extract in pyrogen free 0.89% NaCl saline solution, sterilized by filtering through 0.22 µm pore diameter filter and then diluted to different working concentrations. The endotoxin level in the purified PGe solutions was examined by Pyrotell® Limulus amebocyte lysate assay kit (Associates of Cape Cod, East Fal- mouth, MA, USA) according to the manufacturer's instructions.

Minimal inhibitory concentration and minimal bactericidal concentration of Cephalexin (Ceph) with PGe

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by a microdilution method in 96-well plates following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2013). *Staphylococcus aureus* ATCC 29213 was used as control. Briefly, bacteria were activated from frozen stocks by overnight culture at 37°C on Trypticase Soy Agar (TSA) (Britania, Buenos Aires, Argentina) under aerobic conditions. Then, bacterial growth was diluted in sterile ultrapure water to reach a density of 0.5 McFarland standard corresponding to 1.0×10^8 colony forming units (CFU)/mL. To obtain different concentrations of the antibiotic serial 1:2 dilutions were prepared in Müeller-Hinton broth (Laboratorios Britania S.A, Buenos Aires, Argentina) from the working solution. Then, PGe was incorporated to a final concentration of 0.5 and 3 mg/mL per well to each antibiotic concentration. Activated bacteria were added at a final concentration of 1×10^5 CFU per well. Negative controls (wells without PGe) and positive (viability) controls (wells without cephalexin) were included. After incubation for 24 h at 37°C, plates were evaluated for the visual presence or absence of microbial growth, and with a spectrophotometer (Microplate Reader, SPECTROstar^{Nano}, BGM / LABTECH) by monitoring absorption at 600 nm. Experiments were done in triplicate and the MIC was defined as the lowest concentration at which no

visible growth was observed. In all experiments performed, optical densities (OD) greater than 1 corresponded to visible microbial growth.

Minimum bactericidal concentration (MBC) was determined by inoculating from negative growth wells in the MIC assay onto sterile Müeller-Hinton agar (Laboratorios Britania S.A, Buenos Aires, Argentina). After incubation at 37°C for 24 h, the bacterial colonies on the plates were counted. The lowest concentration of Ceph with PGe which prevented growth and reduced the starting inoculum by 99.9% was defined as the MBC.

Random distribution and intramammary application procedure

Random sampling using computer generated random numbers was used to allocate the animals to either of the treatments.

Intramammary inoculation of different formulations was performed as follows: teat ends were swabbed with 70º alcohol and then the tip of the syringe nozzle of the IM infusions was inserted into the teat canal. Following infusion, the teat was massaged in a dorsal direction. All teats were dipped in 1% iodine teat dip. In PGe + Ceph group, two different syringes were applied in succession.

All procedures used in this study were approved by the Ethics and Security Committee of the Facultad de Ciencias Veterinarias, UNL and were consistent with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGlone, 2010).

Sampling procedures, isolation and identification of microorganisms

Quarter foremilk samples (~5 mL) were collected aseptically using standard procedures (Oliver *et al.*, 2004) from all cows at the day of the last milking prior to treatment administration (pre-drying off samples) and within 24 h after calving (post-calving samples). Quarter samples were immediately refrigerated until culture was carried out.

Microbiological identification of milk samples taken at both periods (pre-drying off and post-calving) was performed according to standard procedures (Oliver *et al.*, 2004). Each sample was incubated aerobically onto 5% blood-agar plates at 37ºC and examined after 24 and 48 h. Briefly, *Staphylococci* were presumptively identified based on colony

morphology, Gram's stain, catalase test and hemolysis on blood agar. *Staphylococcus aureus* and coagulase-positive staphylococci were differentiated from non-aureus staphylococci (NAS) isolates based on coagulase production using rabbit plasma. *Streptococci* were identified based on Gram-staining, catalase test, CAMP test, hydrolysis of aesculin, hippurate, and growth in 6.5% NaCl broth. Gram-negative bacteria were identified based on Gram staining, oxidase test and presumptive differentiation was carried out in Triple Iron Sugar (TSI) medium and Sulfide Indole Motility (SIM) medium (Britania, Buenos Aires, Argentina). A positive culture was defined when three or more colonies of a single pathogen from a mammary quarter were observed, except for *S. aureus* that presence of one colony was considered as positive. A sample was considered contaminated if three or more colony types were present and were excluded from the study.

Milk yield and somatic cell count

For determination of milk SCC, milk samples were preserved with azidiol (0.3%) at 4ºC and analysed within 24 h by Laboratorio Regional de Servicios Analíticos (Esperanza, Santa Fe, Argentina) using an automated counter (Somacount 300, Bentley Instruments, Minesotta, USA).

References

Oliver SP, Gonzalez RN, Hogan JS, Jayarao BM & Owens WE 2004 Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. Verona WI, USA: 4th Edition, National Mastitis Council

McGlone J 2010 Guide for the care and use of agricultural animals in research and teaching. Federation of Animal Science Societies

Table S1: Generalized linear mixed models (GLMM, with binary logistic link function) results of the effect of treatment (Ceph or PGe + Ceph) on incidence of new dry period IMI.

Bacterial categories	**Fixed effects**	**Level**	**F**	***P*-value**	**Exp. Coefficient (OR)**	**95% CI for OR**
All microorganism	Intercept		0.32	<0.001	6.77	3.86 – 11.85
	Treatment	Ceph (ref.) PGe + Ceph	0.63	0.427	0.75	0.38 – 1.51
	Lactation number	1 or 2 (ref.) 3 or more	0.01	0.901	1.04	0.51 – 2.16
Staphylococcus aureus	Intercept		0.31	<0.001	22.61	9.71 – 52.62
	Treatment	Ceph PGe + Ceph (ref.)	0.62	0.431	0.67	0.24 – 1.81
	Lactation number	1 or 2 3 or more (ref.)	0.006	0.938	1.042	0.37 – 2.94
NAS	Intercept		0.21	<0.001	12.86	6.54 – 25.26
	Treatment	Ceph PGe + Ceph (ref.)	0.11	0.743	0.86	0.37 – 2.02
	Lactation number	1 or 2 3 or more (ref.)	0.29	0.587	1.28	0.51 – 3.21
Enviromental (*Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli*)	Intercept		0.13	<0.001	9.69	5.07 – 18.55
	Treatment	Ceph PGe + Ceph (ref.)	0.01	0.921	1.04	0.46 – 2.36
	Lactation number	1 or 2 3 or more (ref.)	0.25	0.618	0.81	0.35 – 1.86

Contagious (*S. aureus*, *Streptococcus agalactiae* and *Corynebacterium spp*.)	Intercept		1.25	<0.001	20.70	9.16 – 46.77
	Treatment	Ceph	2.13	0.146	0.49	0.19 – 1.27
		PGe + Ceph (ref.)				
	Lactation number	1 or 2	0.30	0.582	1.32	0.49 – 3.57
		3 or more (ref.)				

References: NAS, non-aureus staphylococci; ref: reference category; OR, Odds Ratio; CI, confidence interval. Lactation number was included in the model to control its effect.

Table S2: Generalized linear mixed models (GLMM) with binary logistic link function results of the effect of treatment (Ceph and PGe + Ceph) on bacteriological cure rate during the dry period.

Bacterial categories	**Fixed effects**	**Level**	**F**	***P*-value**	**Exp. Coefficient (OR)**	**95% CI for OR**
All microorganism	Intercept		2.26	0.936	0.97	0.43 – 2.16
	Treatment	Ceph PGe + Ceph(ref.)	0.007	0.932	0.95	0.33 – 2.74
	Lactation number	1 or 2 3 or more(ref.)	4.23	0.042	3.53	1.04 – 11.92
Staphylococcus aureus	Intercept		3.64	0.012	0.075	0.01 – 0.54
	Treatment	Ceph PGe + Ceph(ref.)	6.26	**0.018**	15.4	1.66 – 142.52
	Lactation number	1 or 2 3 or more(ref.)	4.76	0.036	27.75	1.25 – 616.20
NAS	Intercept		1.43	0.027	26.36	1.49 – 466.65
	Treatment	Ceph PGe + Ceph(ref.)	2.71	0.109	0.102	0.006 – 1.71
	Lactation number	1 or 2 3 or more(ref.)	0.82	0.819	0.744	0.05 – 10.17
Enviromental (*Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli*)	Intercept		0.31	0.006	7.43	1.81 – 30.50
	Treatment	Ceph PGe + Ceph(ref.)	0.63	0.431	0.52	0.10 – 2.70
	Lactation number	1 or 2 3 or more(ref.)	0.01	0.89	0.89	0.16 – 4.98

Contagious (*S. aureus*, *Streptococcus agalactiae* and *Corynebacterium spp*.)	Intercept		3.17	0.026	0.16	0.034 – 0.79
	Treatment	Ceph	4.46	0.042	6.99	1.07 – 45.54
		PGe + Ceph(ref.)				
	Lactation number	1 or 2	4.65	0.038	19.54	1.18 – 322.53
		3 or more(ref.)				

References: NAS, non-aureus staphylococci; ref: reference category; OR, Odds Ratio; CI, confidence interval. Lactation number was included in the model to control its effect.

Table S3: Means and 95% Confidence Interval of milk yield and SCC by cows treated with Ceph and PGe + Ceph in relation to the months of lactation.

		Milk production (Liters)			SCC (x 10^3 Cells/mL)		
Month of lactation	**Treatment**	**Averages**	**95% CI**		**Averages**	**95% CI**	
1	Ceph	27.40	25.46	29.35	640.3	426.2	854.3
	PGe+Ceph	27.18	25.50	28.87	912.7	577.2	1248.1
2	Ceph	30.68	28.98	32.39	556.4	357.5	755.4
	PGe+Ceph	30.06	28.39	31.73	656.4	418.9	893.9
3	Ceph	28.56	26.51	30.62	534.6	319.0	750.2
	PGe+Ceph	28.67	26.62	39.71	419.8	293.5	546.1
4	Ceph	24.69	22.93	26.44	490.2	280.0	700.5
	PGe+Ceph	25.99	24.61	27.38	564.2	369.3	759.0
5	Ceph	24.73	22.86	26.60	807.6	526.7	1088.5
	PGe+Ceph	26.01	24.78	27.25	576.6	346.5	806.7
6	Ceph	22.47	20.67	24.26	431.4	281.5	581.3
	PGe+Ceph	24.16	22.79	25.53	625.3	367.6	883.0
7	Ceph	21.48	19.72	23.24	596.2	349.8	842.6
	PGe+Ceph	22.35	20.84	23.85	674.5	424.4	924.7
8	Ceph	18.83	17.15	20.51	592.3	381.6	803.0
	PGe+Ceph	21.01	19.50	22.52	529.9	335.5	724.3
9	Ceph	17.96	16.38	19.54	454.2	246.8	661.6
	PGe+Ceph	19.17	17.75	20.60	649.0	416.4	881.5
10	Ceph	15.60	14.35	16.85	542.4	290.2	794.6
	PGe+Ceph	17.51	16.01	19.00	724.1	424.1	1024.2