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A Survey of Bacterial Pathogens Detected in Feces and Wool in Small Ruminants (Pilot Study)

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Abstract: Sheep feces can carry a high concentration of pathogenic and non-pathogenic bacteria, which potentially may contaminate wool as well as the shearers or wool manipulators through direct contact. A pilot study was carried out to determine the presence of bacterial DNA in feces and the degree of bacterial contamination in wool in two species of ruminants. Fourteen 2-month old lambs and 14 kids (7 male and 7 female), uncastrated, no twins, with their mothers, were randomly selected at weaning from a free flock grazing on naturalized pasture of Los Ríos region, Chile. Fecal and wool samples were taken once and analyzed for genomic DNA of *Salmonella typhimurium* containing the virulence plasmid spv, *Eschrichia coli* serotype O157, *Clostridium perfringens* type C containing α toxin and *Mycobacterium avium* sp paratuberculosis containing the IS*900* insertion element. The results showed that lamb and kids feces had higher contents of bacterial DNA for *E. coli* O157 and SalmT than lamb wool, although only one lamb showed these two bacteria on its wool. The bacterial species influenced the DNA expression for 16S in both, feces (P=0.05) and wool (P=0.0006) and for *E. coli* O157 and SalmT only in feces (P<0.0001). The sex was associated with *E. coli* detection in lambs feces (P<0.0007) and in kids feces (P<0.05). The values obtained for MAP IS*900* and Cpa DNA contents, considering both species and sex, were undetectable. In conclusion, lamb and kids feces should potentially contaminate wool especially by *Eschrichia coli* O157 and *Salmonella typhimurium*, representing a potential health risk and public health concern, especially for shearers and wool handlers.

Keywords: Genomic DNA, Bacteria, Lambs, Kids

1. Introduction

Ovine feces are known to carry a large range of microbial indicators and pathogens (Moriarty et al. 2011[b]), which can survive for a long time on pasture [1], slurry and soil [2-4]. Cattle are considered as an important reservoir of *E. coli* serotype O157:H7 (*E. coli* O157) for humans, however, small ruminants may also shed bacterial pathogens to humans [5-7]. Examples the latter are: *E. coli* O157 [7, 8], *Clostridium difficile* [9], *Campylobacter sp* [7, 10], *Salmonella sp* [7], *Brucella mellitensis* or *Coxiella burnetti* [11]. Sheep and goats can also serve as reservoirs for numerous gastrointestinal pathogens [12-14], of *Mycobacterium avium subsp. paratuberculosis* (MAP), a pathogen that infects and affects

primarily domestic ruminants, being sheep less susceptible to infection than goats [15] and young individuals more susceptible than old animals [16]. The presence of all the above mentioned pathogens in ruminants, in terms of risk for the public health, is not only associated to animal consumption, but also to manipulation of pathogen's contaminated animal products, such as wool, both, before and after washing [8].

The aim of the present study was to determine the presence of *E. coli* O157, *Salmonella enterica* subgroup enterica serotype typhimurium (SalmT), *Clostridium perfringens* Type C (Cpa) and MAP in feces and wool at the weaning period among grazing lambs and kids at weaning in grazing lambs and kids and its association with animal sex, considering the different management and normal behavior that they have, as a possible public health problem.

2. Materials and Methods

2.1. Ethics

All experimental animal procedures followed the principles of the Guide for Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethical Committee of Guangdong Institute of Applied Biological Resources.

2.2. Animal Samples

The sampling was carried out in a farm located to 7 km far from Paillaco city, Los Ríos region, Chile (40.1° S and 72.8° W), during December 2015. Fourteen 2-month old lambs and kids (seven un-castrated male and seven female) no twin and their mothers, were randomly selected from a large free-flock (vaccinated against clostridial diseases oncea year, plus antiparasitics twice a year), grazing on naturalized pasture until weaning, moment in which the pasture composition was measured (32% DM, 6.18% CP, 1.19% EE, 0.75 Mcal kg^{-1} ME, 25.79% NDF, 14.49% ADF). The day before weaning, samples of feces were taken directly from the rectus. Wool was taken at the loin height. Samples were kept in Eppendorf tubes and transported on ice to the Institute of Biochemistry and Microbiology, belonging to the Universidad Austral de Chile to their storage at -80°C until further analysis. Fecal (approximately 0.5 g) and wool (approximately 8 fibers of the same longitude/animal) samples were taken and submitted to a DNA extraction protocol, using 300 μL Chelex-100 5%, adding 2μL lysozyme (10 mg/mL) for feces, and 2μL proteinase K for wool samples. The extracted DNA was kept at -80 ºC until its analysis. The bacterial culture for each of the four bacteria considered in this study was provided by the Institute of Biochemistry and Microbiology to be used as positive controls. DNA extraction from bacterial culture was obtained using E. Z. N. A and the tissue DNA kit D3396-02, OMEGA-BIOTEK, except in the case of MAP which was extracted according to the protocol described by Salgado *et al* (2014).

2.3. Genomic DNA

The following complete coding sequences were obtained from different sources: 16S bacterial ribosomal subunit DNA [17], *E. Coli* O157 [18, 19], Cpa [20] and MAP IS*900* [21]. For SalmT, the coding sequence was retrieved from the GenBank database (GenBank accession number 383494824). The primer design (Table 1) was developed using AmplifX 1.5 software for SalmT, *E. coli*, Cpa, and MAP IS*900* DNA. The reaction was performed in a StepOnePlus Real-Time PCR System using 5 μL Green Master Mix Promega, with1 μL of sample as template, and 0.5 μL of each primer (10 μM), following a standard protocol with an initial denaturation to 95ºC for 10 min and then 40 cycles to 60°C. The obtained Ct values were used to obtain the concentration from a calibration curve prepared with different amounts of DNA from positive controls. The initial DNA concentrations to positive controls of *E. coli* O157, SalmT, MAP IS*900* and Cpa, measured by spectrophotometry were 16.23, 73.00, 57.00 and 48.00 ng/μL, respectively. Each DNA control was serially diluted 7 times in proportions 1:5. The slopes from efficiency curves for dilutions of each bacterium DNA (*E. coli* O157, SalmT, MAP *IS900* and Cpa) with its respective primers were -3.428 (r^2: 0.99), -3.581 (r^2: 0.99), -3.531 (r^2: 0.99) and -3.718 (r^2: 1.00), respectively. Arbitrary, Ct values > 35 were considered negative. In order to detect pathogens in fecal and wool of small ruminants, descriptive and quantitative statistics were used. LSM ± SEM were estimated using GraphPad Prism statistical software version 5.03. T-test was performed to assess significant differences between means at P-value ≤ 0.05.

Table 1. Primer specifications.

Gene	Sequence (5′to 3′)	Length	Temp	Effi
16S-F	ACTCCTACGGGAGGCAGCAGT	180bp	60º	100.72%
16S-R	TATTACCGCGGCTGCTGGC			
SalmT-F	AGTACGGCCGCAAGGTTAAA	150bp	65º	90.21%
SalmT -R	AGTTCCCGAAGGCACAAATC			
E.coli O157-F	GTACAAGTCCACAAGGAAAG	125bp	61	95.75%
E.coli O157- R	CTTGTTTCGATGAGTTTATCTGCA			
Cpa-F	GCTAATGTTACTGCCGTTGA	109bp	63º	85.75%
Cpa-R	CCTCATTAGTTTTGCAACC			
MAP IS900-F	CCGCTAATTGAGAGATGCGATT	115bp	64º	91.96%
MAP IS900-R	CCAGACAGGTTGTGCCACAA			

16S: 16s ribosomal subunit 16S; Salm: Salmonella Typhimurium (containing the virulence plasmid-spv); *E.coli* O157: Escherichia coli serotype O157; CPA Clostridium perfringens type C containing toxin α; MAP IS900: Mycobacterium avium sp para-Tbc containing its insertion element IS900

3. Results and Discussion

The risk for people to become infected by zoonotic agents from small ruminants varies depending on the infectious agent, as well as the way humans handle the animals [11]. Ruminant feces can carry high concentration of pathogenic and non-pathogenic gastrointestinal bacteria [12-14], constituting a risk factor not only associated to consumption, but mainly associated to the handling of pre and post washed wool. This affects not only the wool itself, but also the shearers and wool handlers through direct contact.

Table 2 shows the bacterial DNA quantification detected in feces and wool from kids and lambs according to sex. As expected, feces showed higher content of 16S bacterial DNA, *E. coli* O157 and SalmT than wool. Instead of the higher presence of *E. coli* O157 in feces from female lambs and kids, these bacteria were only detected in one female lamb in the same amount than in feces. SalmT was detected in 4 lambs and 3 kids feces (independent to sex). In wool, this bacteria was found only in one female lamb, the same female that tested positive to *E. coli* O157 in wool. This diagnosis can be explained by the grooming behavior among them, because this animal did not show SalmT in feces. Regarding MAP presence and Cpa, they were not detected in feces or wool.

Table 2. Individual data of total bacterial genomic DNA (ng/µL) in feces and wool of lambs and kids according to sex (Descriptive data).

Lamb Feces						Lamb Wool				
Lambs	16S	E.coli	Salm	MAP	Cpa	16s	E.coli	Salm	MAP	Cpa
Female (n=7)	0.753	0.013	0.000			0.020	0.000	0.000		
Male (n=7)	1.288	0.090	0.001			0.021	0.000	0.000		
Kid Feces						Kid Wool				
Kids	16S	E.coli	Salm	MAP	Cpa	16s	E.coli	Salm	MAP	Cpa
Female (n=7)	1.601	0.009	0.000			0.006	0.000	0.000		0
Male (n=7)	1.961	0.004	0.000			0.002	0.000	0.000		

DNA concentrations to calibration curves for 16S, E.coli O157, SalmT, MAP IS*900* and Cpa ranged between 86-0.01, 16.23-0.01, 73-0.1, 57-0.02, 48-0 ng/µL, respectively

The quantification of total genomic DNA (ng/µL) detected in feces and lamb wool according to bacterial species and sex is shown in Table 3. The DNA expression for 16S was lower in lamb than kids feces (P=0.05), however lambs showed higher genomic DNA contents for 16S than kids wool (P=0.0006). The DNA expression for *E. coli* O157 and SalmT were higher in lambs than kids feces (P<0.0001). In wool, *E. coli* O157 detection was undeterminable between species and significant for SalmT (P<0.001).

Table 3. Quantification of total bacterial genomic DNA (ng/µ) detected in feces and wool in lambs and kids according to sex (LSM±SEM).

Bacterial DNA (ng/ul) Between bacterial species*	Lamb feces (n=7)	Lamb wool (n=7)	P	Kids feces (n=7)	Kids wool (n=7)	P
16S	1.02 ± 0.15	0.02 ± 0.00	<0.0001	1.78 ± 0.26	0.00 ± 0.00	<0.0001
E.coli	0.04 ± 0.02	0.00 ± 0.00	<0.0001	0.01 ± 0.00	0.00 ± 0.00	ud
SalmT	0.03 ± 0.00	0.00 ± 0.00	<0.0001	0.00 ± 0.00	0.00 ± 0.00	<0.0001
Lambs between sex**	Male Lambs Feces (n=7)	Wool (n=7)	P	Female Lambs Feces (n=7)	Wool (n=7)	P
16S	0.75 ± 0.16	0.02 ± 0.00	<0.0001	1.29 ± 0.21	0.02 ± 0.00	<0.0001
E.coli	0.01 ± 0.00	0.00 ± 0.00	<0.0001	0.09 ± 0.01	0.00 ± 0.00	ud
SalmT	0.02 ± 0.01	0.00 ± 0.12	=0.0002	0.00 ± 0.00	0.00 ± 0.00	<0.0001
Kids between sex***	Male Kids Feces (n=7)	Wool (n=7)	P	Female Kids Feces (n=7)	Wool (n=7)	P
16S	1.60 ± 0.30	0.01 ± 0.00	<0.0001	1.96 ± 0.44	0.00 ± 0.00	<0.0001
E.coli	0.01 ± 0.00	0.00 ± 0.00	ud	0.01 ± 0.00	0.00 ± 0.00	ud
SalmT	0.03 ± 0.01	0.01 ± 0.00	<0.001	0.00 ± 0.00	0.00 ± 0.00	ns

*For 16s: P value between spp (Feces) (P=0.05); P value between spp (wool) (P=0.0006); For E.coli: P value between spp (Feces) (P<0.0001); P value between spp (wool) (P=ud); For Salm3: P value between spp (Feces) (P<0.0001); P value between spp (wool) (P<0.001)

**For 16s: P value between spp (male vs. female Feces) (P=ns); P value between spp (male vs. female wool) (P=ns); For E.coli: P value between spp (male vs. female Feces) (P<0.0007); P value between spp (male vs. female wool) (P=ud); For Salm3: P value between spp (male vs. female Feces) (P=ns); P value between spp (male vs. female wool) (P=ns)

***For 16s: P value between spp (male vs. female Feces) (P=ns); P value between spp (male vs. female wool) (P=ns); For coli: P value between spp (male vs. female Feces) (P=ns); P value between spp (male vs. female wool) (P=ud); For Salm3: P value between spp (male vs. female Feces) (P<0.05); P value between spp (male vs. female wool) (P=ns)

In this study, the main bacterial DNA found in feces and wool in kids and lambs was *E. coli* O157 and secondary, SalmT. Considering that the quantification of total bacterial genomic DNA (ng/µL) detected in pastures was 0.01 ± 0.04 for 16S, and 0.00 ± 0.00 for all bacteria considered in this study, it may be inferred that bacteria did not come from pastures, therefore, those bacteria came from the animal´s gut.

E. coli can be used as an indicator of water fecal

contamination, however water also can be a source of *Salmonella* [22]. However, the way carcass are contaminated is not clear [23]. Certainly, the prevalence of *E. coli* O157 can be reduced by avoiding the fecal contamination. An adult sheep can show *E. coli* concentrations of 1.62×10^7 per g of fresh feces, being higher in spring/summer than in winter [1, 24]. Munsi et al. [25] identified that the bacterial species in feces from 20 diarrheic sheep had the ratio 30% to *E. coli* and 60% to *Proteus mirabilis*.

In the present study, *E. coli* O157 was detected only in lamb and kids feces. Mersha et al. [8] studied feces, skin and carcasses samples collected from sheep and goats from an export abattoir, detecting *E. coli* O157:H7 in most of them, with no significant differences before and after washing. Reid et al. [26] reported that the main source of carcass contamination was the skin, which can contaminate wool. Petkovsek et al. [27] found that *E. coli* isolates from the skin and soft tissues infection showed a high potential virulence similar to *E. coli* isolates described in urinary infections and bacteremia. In humans, this bacteria has been found as causative agent of neonatal omphalitis [28], necrotizing fasciitis [29] and also infections after burning [30]. Therefore, feces from lamb and kids can potentially contaminate wool, especially with *E. coli* and SalmT, representing an important zoonotic risk for shearers and wool handlers.

Regarding Cpa, the differences between feces and wool found within species or between species were undeterminable, being consistent with the results reported by Gkiourtzidis et al. [31], showing Cpa (which contains the α-toxin) was not found in feces. Regarding MAP IS*900*, the differences between feces and wool found within species were non significant and undeterminable, respectively.

In the present study no significant differences between sex for 16S DNA expression in feces or wool were found (P>0.05). In feces, *E. coli* O157 detection was higher in female than male lambs (P<0.0007), without any significant difference between female and male kids (P>0.05). The SalmT detection was higher in male than female kids (P<0.05) but not between male and female lambs (P>0.05). In wool the DNA expression for *E. coli* O157 was undeterminable between sex in lambs, which was not significant between sex in kids. SalmT detection was not significant between in sex. Instead, there are no publications related to bacterial pathogens and sex, Cong et al. [32] found a change in the bacterial composition according to sex (more amount of Clostridiates, and less of Enterobacteria in female than male in the gastrointestinal tract during early age and breastmilk feeding type (kids fed mother's own breastmilk showed a higher abundance in Clostridiates and Lactobacillus than those fed non mother´s own breastmilk).

4. Conclusion

In the present study we found the lamb and kids feces had higher contents of bacterial DNA for *E. coli* O157 and SalmT than lamb wool, although only one lamb showed these two bacteria on its wool. The bacterial species influenced the DNA expression for 16S in both, feces (P=0.05) and wool

(P=0.0006) and for *E. coli* O157 and SalmT only in feces (P<0.0001). The sex was associated with *E. coli* detection in lambs feces (P<0.0007) and in kids feces (P<0.05). The values obtained for MAP IS*900* and Cpa DNA contents, considering both species and sex, were undetectable. It was concluded that lamb and kids feces could potentially contaminate wool with *E. coli* (particularly in female lambs) and SalmT (especially in male lambs and kids), representing an important health risk for shearers and wool handlers, which must be considered as a public health concern. Further studies should be focused on finding effective measures to address different levels of infection in alive animals to control the direct skin contamination (because prevention is not possible due to the normal animal behavior) as a way to minimize the human zoonotic infection risk.

Conflict of Interest

The authors declare that they have no competing interests

Acknowledgements

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