Population structure of rumen *Escherichia coli* associated with subacute ruminal acidosis (SARA) in dairy cattle

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ABSTRACT

Previous studies indicated that only subacute ruminal acidosis (SARA), induced by feeding a high-grain diet, is associated with an inflammatory response and increased abundance of *Escherichia coli* in the rumen. We hypothesized that ruminal *E. coli* in grain pellet-induced SARA carried virulence factors that potentially contribute to the immune activation during SARA. One hundred twenty-nine *E. coli* isolates were cultured from the rumens of 8 cows (4 animals per treatment) in which SARA had been nutritionally induced by feeding a high-grain diet (GPI-SARA) or a diet containing alfalfa pellets (API-SARA). The population structure of the *E. coli* was evaluated with the ABD genotyping system and repetitive sequence-based (rep)-PCR fingerprinting. Twenty-five virulence factors were evaluated with PCR. *Escherichia coli* numbers were higher in the GPI-SARA treatment than in the API-SARA treatment. The genetic structure of the *E. coli* was significantly different between SARA challenge models. Isolates from GPI-control (46%), API-control (70%), and API-SARA (53%) were closely related and fell into one cluster, whereas isolates from GPI-SARA (54%) grouped separately. The ABD typing indicated a shift from an A-type *E. coli* population to a B1-type population only due to GPI-SARA. Of the 25 virulence factors tested, curli fiber genes were highly associated with GPI. Curli fibers were first identified in *E. coli* mastitis isolates and are potent virulence factors that induce a range of immune responses. Results suggest that under low rumen pH conditions induced by a grain diet, there is a burst in the number of *E. coli* with virulence genes that can take advantage of these rumen conditions to trigger an inflammatory response.

Key words: cattle, subacute ruminal acidosis, *Escherichia coli*, virulence genes

INTRODUCTION

Subacute ruminal acidosis is a metabolic disease in high-producing dairy cattle that results in reductions in feed intake and milk production, and has been defined as a disorder associated with low rumen pH (Plaizier et al., 2008). During low rumen pH, gram-negative bacteria lyse more rapidly, increasing the concentration of LPS in the rumen (Nagaraja and Titgemeyer, 2007). Low pH, high osmotic pressure, and the presence of LPS in the rumen fluid are associated with the development of microlesions and parakeratosis in the rumen epithelium (Kleen et al., 2003). A reduction in barrier function of the rumen epithelium potentially allows LPS to translocate into blood and stimulate an inflammatory response (Plaizier et al., 2008; Khafipour et al., 2009a,b). When SARA was induced by feeding grain pellets (GPI-SARA) or by feeding alfalfa pellets (API-SARA), both diets resulted in low rumen pH and increased rumen osmolarity and LPS but only the GPI-SARA resulted in an inflammatory response (Khafipour et al., 2009a,b). Thus, low rumen pH and high osmolarity by themselves are not enough to explain an inflammatory response.

Molecular microbial analysis of the rumen microbiome demonstrated significant differences in the microbial composition of the rumen with these 2 diets, particularly in the Firmicutes and Bacteroidetes phyla (Khafipour et al., 2009c). Of particular note was the increased abundance of *Escherichia coli* in the GPI-SARA group. In this manuscript we evaluated the genetic composition of these *E. coli* and the virulence factors they carry during GPI- vs. API-SARA.

MATERIALS AND METHODS

Animal experiments were conducted at the University of Manitoba Glenlea Dairy Research Unit in accordance with the guidelines of the Canadian Council on Animal Care (1993). The nutritional models, diet composition, and animal management have been described previously (Khafipour et al., 2009a,b). In brief,

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SARA was nutritionally induced in 4 rumen-cannulated dairy cows by feeding a high-grain diet (grain-pellet challenge: **GPI**) or a diet containing alfalfa pellets (alfalfa-pellet challenge: **API**) in 2 separate experiments (total of 8 animals). In both experiments, animals were fed once daily at 0900 h. In the GPI experiment, cows received a basal diet with a forage-to-concentrate ratio of 50:50. The ration consisted of 25% as alfalfa silage, 25% as barley silage, 40% as an energy supplement, and 10% as a protein supplement. Subacute ruminal acidosis was induced by replacing 21% of the dietary DM with wheat-barley pellets. In the API experiment, cows received a basal diet that consisted of 50% of the DM as concentrate and 50% as chopped alfalfa hay. The concentrate fraction consisted of 39% as an energy supplement, 4.5% as a protein supplement, and 6.5% as roasted soybeans. Subacute ruminal acidosis was induced by replacing 42% of the alfalfa hay DM with alfalfa pellets without changing the forage-to-concentrate ratio or the dietary content of starch.

Thirty-two rumen fluid samples were collected immediately before and at 6 h after feeding during the control (GPI-control and API-control) and SARA periods. Rumen pH was monitored continuously using indwelling pH probes (Khafipour et al., 2009a). Average rumen pH and time spent below pH 5.6 were 6.17 and 118 min/d, respectively, in GPI-control; 6.35 and 112 min/d, respectively, in API-control; 5.97 and 279 min/d, respectively, in GPI-SARA; and 5.85 and 510 min/d, respectively, in API-SARA (Khafipour et al., 2009a, b). For the analysis of *E. coli*, rumen samples were strained through 4 layers of sterile cheesecloth, mixed with an equal volume of 50% sterile glycerol in a 15-mL screw-cap polypropylene tube and stored at -80°C until used for enumeration and isolation of *E. coli*.

Isolation and Enumeration of *E. coli*

Glycerol stocks were thawed on the bench top and *E. coli*/coliform chromogenic medium (CM0956, Oxide Inc., Nepean, ON, Canada) was used for enumeration. Buffered peptone water (pH 7.2, Difco Laboratories, Detroit, MI) was used to make decimal dilutions in 2-mL deep-well plates. Plates were covered with aluminum foil and sterilized at 121°C and 103.4 kPa for 15 min before use. A 100- μL sub-sample of the thawed glycerol stocks was inoculated into 900 μL of 2% buffered peptone water and serially diluted to 10^{-7} . Dilutions from 10^{-1} to 10^{-7} were plated onto medium by dispensing 10 droplets of 10 μL each (Herigstad et al., 2001). Following adsorption of the droplets onto the medium, plates were inverted and incubated at 39°C for 18 h. *Escherichia coli* (purple colonies) were counted at

each dilution that was not overgrown. An appropriate dilution factor was applied to determine the number of *E. coli* per 1 mL of undiluted rumen fluid (cfu/mL).

From plates with at least 10 colonies, 5 putative *E. coli* colonies were picked. Isolates were then purified by repeated streaking on *E. coli*/coliform chromogenic medium. The identities of *E. coli* colonies were reconfirmed by a PCR assay specifically targeting type I fimbriae of *E. coli* (Table 1). Single colonies were inoculated into 15 mL of Luria-Bertani broth (LB; Fisher Scientific, Fairlawn, NJ) and grown for 48 h at 37°C . One milliliter of fresh culture was added to 1 mL of 50% glycerol and stored in -80°C . The remainder was used for the DNA extraction.

DNA Extraction

Cells were harvested by centrifugation at $3,000 \times g$ for 15 min. Pelleted cells were resuspended in 570 μL of $0.5 \times$ Tris-EDTA buffer (5 mM Tris and 0.5 mM EDTA, pH 8.0; Fisher Scientific). Thereafter, 30 μL of 10% SDS (Fisher Scientific) and 10 μL of proteinase K (20 mg/mL; Fisher Scientific) were added to each tube and incubated for 1 h at 37°C . Subsequently, 100 μL of 5 M NaCl and 80 μL of 10% cetyl trimethylammonium bromide were added to the tubes, mixed thoroughly by hand and incubated for 10 min at 65°C . Then, 600 μL of phenol:chloroform:isoamylalcohol (25:24:1; Fisher Scientific) was added to the tubes, mixed and centrifuged at $10,000 \times g$ at 4°C for 5 min. The supernatants were transferred to fresh tubes, mixed with 600 μL of chloroform:isoamylalcohol (24:1; Fisher Scientific), and centrifuged at $10,000 \times g$ at 4°C for 5 min. Supernatants were transferred again to fresh microfuge tubes and mixed with equal volumes of isopropanol (Fisher Scientific) to precipitate nucleic acid. The tubes were left on ice for 15 min and then centrifuged at $10,000 \times g$ for 10 min. Then, pellets were washed with 500 μL of 70% ethanol for 5 min and subsequently centrifuged at $10,000 \times g$ for 10 min. The resulting nucleic acid pellets were redissolved in 100 μL of $0.5 \times$ Tris-EDTA buffer (pH 8.0) and stored at -20°C until further analysis. The DNA concentration and purity were determined spectrophotometrically by measuring the optical density and the ratio of the absorbance at wavelength 260 and 280 nm (Beckman Coulter, DU/800 UV/VIS, Beckman Coulter Inc., Fullerton, CA).

Rep-PCR DNA Fingerprinting

Repetitive sequenced-based (**rep**)-PCR DNA fingerprinting with BOX A1R primer was used to differentiate *E. coli* isolates (Dombek et al., 2000). The method targets the interspersed, conserved, repetitive DNA

Table 1. Primers used in this study

Target ¹	Primers	Primer sequence (5' to 3') ²	Fragment size (bp)	Annealing temp (°C)	Reference
<i>Escherichia coli</i> virulence genes					
SPATE	SPATE1	GAGGTCAACAACCTGAACAAACGTATGGG	617	57	Kotlowski et al., 2007
	SPATE2	CCGGCACGGGCTGTCACTTTCCAG			
<i>agn43</i>	Ag43F	TGACACAGGCAATGGACTATGACCG	317	67	Kotlowski et al., 2007
	Ag43R	GGCATCATCCCGGACCGTGC			
<i>aggR</i>	aggRf	GAGTTAGGTCACTCTAACGCAGAGTTG	377	61	Kotlowski et al., 2007
	aggRr	GACCAATTCCGGACAACCTGCAAGCATCTAC			
<i>aidA</i>	AIDA1	TATGCCACCTGGTATGCCGATGAC	545	69	Kotlowski et al., 2007
	AIDA2	ACGCCCACATTTCCCCAGAC			
<i>PAI I</i>	PAI1	TAGCTCAGACGCCAGGATTTTCCCTG	736	61	Kotlowski et al., 2007
	PAI2	CCTGGCGCCTGCGGGCTGACTATCAGGG			
<i>pap</i>	PapF	CCGGCGTTTCAGGCTGTAGCTG	97	65	Kotlowski et al., 2007
	PapR	GCTACAGTGGCAGTATGAGTAATGACCGTTA			
<i>sfaD-E</i>	SfaF	CGGAGGAGTAATTACAAACCTGGCA	408	64	Martin et al., 2004
	SfaR	CTCCGGAGAAGTGGGTGCATCTTAC			
<i>afaE</i>	AfaF	TATGGTGAGTTGGCGGGGATGTACAGTTACA	271	58	Kotlowski et al., 2007
	AfaR	CCGGGAAAGTTGTCTGGATCCAGTGT			
<i>cnf</i>	CnfF	AGTACTGACACTCACTCAAGCCGC	930	62	Kotlowski et al., 2007
	CnfR	GCAGAACGACGTTCTTCATAAGTATCACC			
<i>vt1</i>	Vt1F	CGCATAGTGGAACCTCACTGACGC	91	64	Kotlowski et al., 2007
	Vt1R	CATCCCCGTACGACTGATCCC			
<i>vt2</i>	Vt2F	CGGAATGCAAATCAGTCGTCACCTCAC	265	65	Kotlowski et al., 2007
	Vt2R	TCCCCGATACTCCGGAAGCAC			
<i>hlyA</i>	HlyAF	TGCAGCCTCCAGTGCATCCCTC	355	63	Kotlowski et al., 2007
	HlyAR	CTTACCACTCTGACTGCGATCAGC			
<i>eae</i>	EaeF	CCAGGCTTCGTACAGTTGCAGGC	300	66	Kotlowski et al., 2007
	EaeR	CGCCAGTATTCCGCCACCAATACC			
<i>bmaE</i>	BmaEF	CTAACTTGCCATGCTGTGACAGTA	302	59	Martin et al., 2004
	BmaER	TTATCCCCTGCGTAGTTGTGAATC			
<i>fliC</i>	FliC1	CAAGTCATTAATACMAACAGCC	900–2,600	55	Machado et al., 2000
	FliC2	GACATRTTRGAVACTTCSGT			
<i>crl</i>	Crl1	TTTCGATTGTCTGGCTGTATG	250	58	Ewers et al., 2007
	Crl2	CTTCAGATTCAGCGTCGTC			
<i>cgsA</i>	CgsAf	ACTCTGACTTGACTATTACC	200	55	Maurer et al., 1998
	CgsAr	AGATGCAGTCTGGTCAAC			
<i>focG</i>	FocGf	CAGCACAGGCAGTGGATACGA	364	63	Johnson and Stell, 2000
	FocGr	GAATGTCGCGCTGCCATTGCT			
<i>neuC</i>	NeuC1	GGTGGTACATTCCGGGATGTC	676	58	Ewers et al., 2007
	NeuC2	AGGTGAAAAGCCTGGTAGTGTG			
<i>kpsMT II</i>	KpsMTII f	GCGCATTTGCTGATACTGTTG	280	58	Ewers et al., 2007
	KpsMTII r	CATCCAGACGATAAGCATGAGCA			
<i>ompA</i>	OmpAF	AGCTATCGCGATTGCAGTG	919	58	Ewers et al., 2007
	OmpAR	GGTGTGCGCAGTAACCGG			
<i>ompC</i>	OmpCF	GCAGGCCCTTTGTTCGATA	1,236	59	Oteo et al., 2008
	OmpCR	GCCGACTGATTAATGAGGGTTA			
<i>usp</i>	Usp1	ATGCTACTGTTTCCGGGTAGTGTGT	1,000, 2,500, 3,000	68	Nakano et al., 2001
	Usp2	CATCATGTAGTCGRGGCGTAACAAT			
<i>malX</i>	MalXF	GGACATCCTGTTACAGCGCGCA	922	58	Ewers et al., 2007
	MalXR	TCGCCRCCAATCACAGCCGAAC			
<i>E. coli</i> fimbriae subunit					
<i>fimH</i>	FimHf	CTGGTCATTTCGCCTGTAAAACCGCCA	846	68	Kotlowski et al., 2007
	FimHr	GTCACGCCAATAATCGATTGCACATTCCCT			
Rep-PCR DNA fingerprinting					
—	BOX A1R	CTACGGCAAGGCGACGCTGACG	—	53	Dombek et al., 2000
<i>E. coli</i> ABD typing					
<i>chuA</i>	ChuAf	CGGACGAACCAACGGTCAGGAT	281	70	Kotlowski et al., 2007
	ChuAr	TGCCGCCAGTACCAAAGACACG			
<i>tsp</i>	Tsp1	GGGAGTAATGTCTGGGGCATTTCAG	161	70	Kotlowski et al., 2007
	Tsp2	CATCGCGCCAACAAAGTATTACGCAG			
<i>yja</i>	Yjaf	CGTGAAGTGTCTCAGGAGACGCTGC	226	70	Kotlowski et al., 2007
	Yjar	TGCGTTCTCTCAACCTGTGACAAACC			

Continued

Table 1 (Continued). Primers used in this study

¹SPATE = genes encoding serine protease autotransporter; *agn43* = the gene coding for antigen involved in *E. coli* autoaggregation; *aggR* = the gene for adhesin of aggregative adherence fimbriae I; *aidA* = adhesin involved in diffuse adherence; *PAI I* = pathogenicity island I; *pap* = *E. coli* Pap pili subunit and a part of pathogenicity island II; *sfa* = the gene coding for S-fimbriae minor subunit; *afaE* = gene encoding AfaE-III afimbrial adhesin involved in *E. coli* diffuse adherence; *cnf* = cytotoxic necrotizing factors Cnf1 and Cnf2; *vt1* and *vt2* = shiga toxins (verocytotoxins) 1 and 2; *hlyA* = α hemolysin; *eae* = intimin; *bmaE* = gene for M-agglutinin subunit; *fliC* = flagellin; *crl* = curli regulatory gene; *cgsA* = curli structural gene which encodes curlin subunit; *focG* = F1C fimbriae; *neuC* = K1 capsular polysaccharide; *kpsMTII* = group II capsule antigens; *ompA* = outer membrane protein A; *ompC* = outer membrane protein C; *usp* = uropathogenic-specific protein; *malX* = pathogenicity-associated island marker for UPEC CFT073; *fimH* = gene encoding for adhesive subunit of *E. coli* type I fimbriae; *chuA* = the gene that is required for heme transport in *E. coli* O157:H7; *tsp* = the gene coding for a putative DNA fragment (TSPE4.C2) in *E. coli*; *yja* = gene coding for a protein of unknown function.

²R = G or A; V = A or C or G.

sequences in bacterial genome, such as REP, ERIC, or BOX elements, depending on the primer used (Mohapatra et al., 2007). Because these repetitive units are highly conserved, selective amplification of distinct genomic regions between these repetitive sequences generates unique strain-specific fingerprint patterns that can be used to differentiate *E. coli*.

Each 20.5- μ L rep-PCR reaction included 2.0 μ L of 10 $\times$ EconoTaq buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100; Lucigen Corporation, Middleton, WI), 1 μ L of 25 mM MgSO₄ (Fisher Scientific), 0.4 μ L of 10 mM nucleotide mix (Fisher Scientific), 0.2 μ L of BOX A1R primer (25 mM; Table 1), 1 U EconoTaq polymerase (Lucigen Corporation), 16.2 μ L of H₂O, and 0.5 μ L of DNA (approximately 50 ng). A control reaction mixture containing 0.5 μ L of Mili-Q water (Millipore water purification system, Millipore Corp., Billerica, MA) instead of template DNA was included in each set of PCR reactions. Amplification was performed in a Techne-Genius thermocycler with an initial denaturation step of 94°C for 2 min; then 30 cycles of 94°C for 3 s, 92°C for 30 s, 53°C for 1 min, and 65°C for 3 min; followed by a single-step extension at 65°C for 8 min (Dombek et al., 2000). The PCR products were separated on 1.5% agarose (Promega, Madison, WI) gel and stained in a 0.5- μ g/mL ethidium bromide solution (Fisher Scientific) for 30 min. The gel images were captured with a FluorChem SP (Alpha Innotech Corp., San Leandro, CA). The DNA fingerprints were then analyzed for the presence or absence of 23 unique bands ranging from 420 to 3,300 bp by FluorChem SP (ver. 5.0) software, which resulted in a binary matrix that was subjected to cluster analysis. The positions of bands were normalized with external (1-kb DNA ladder) and internal standards (3 or 4 common bands between most of *E. coli* isolates) (Dombek et al., 2000).

ABD Typing

Escherichia coli isolates were assigned to 1 of the 4 groups (A, B1, B2, and D) using the methods of Clermont et al. (2000) and Kotlowski et al. (2007).

The method uses 2 genes (*chuA* and *yjaA*) and a DNA fragment (TSPE4.C2) as markers for phylogenetic grouping of isolates (Table 1). In brief, isolates belonging to A or B1 groups are negative for the *chuA* gene. The *chuA*-negative isolates are separated into TSPE4.C2 fragment-negative (A) or TSPE4.C2-positive (B1) strains. The *chuA*-positive strains are further classified if they are *yjaA*-positive (B2), or *yjaA*-negative (D).

Virulence Genes

A set of 25 virulence genes (Table 1), reported in the literature to be associated with intestinal and extraintestinal virulent phylotypes of *E. coli*, including enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, diffusely adherent *E. coli*, avian pathogenic *E. coli*, and uropathogenic *E. coli* (UPEC), was used in this analysis. These genes encode for a range of virulence factors, including adhesins, aggregation factors, toxins, pathogenicity islands, autotransporters, and capsule synthesis.

The PCR reactions contained 50 ng of chromosomal DNA, 5 μ L of 10 $\times$ EconoTaq buffer (Lucigen Corporation), 2 μ L of MgCl₂ (50 mM; Fisher Scientific), 1 μ L of 10 mM nucleotide mix (Fisher Scientific), 1 μ L of each primer (25 pmol), 2 units of EconoTaq polymerase (Lucigen Corporation), and Mili-Q water to 50 μ L. Thermal cycling conditions were: 1 cycle of initial denaturation (95°C, 2 min); 30 cycles of denaturation (94°C, 1 min), annealing (see Table 1; 1 min), and extension (72°C, 2 min); followed by a final extension (72°C, 5 min). The amplified products were subjected to electrophoresis using 1.5% agarose (Promega) gel. Results were presented as percentage and cfu/mL of rumen fluid of *E. coli* isolates carrying virulence genes (percentage $\times$ total cfu/mL).

Statistical Analysis

The rep-PCR DNA fingerprints were clustered using the maximum composite likelihood and the unweighted pair-groups method average clustering methods

Table 2. *Escherichia coli* numbers in the rumen fluid during grain pellet (GPI)- and alfalfa pellet (API)-control, and GPI- and API-SARA feeding challenges

SARA challenge/ Sampling time	Number of <i>E. coli</i> (log ₁₀ cfu/mL) during:			Effect, <i>P</i> -value		
	Control diet ¹	SARA diet	SEM	Diet	Time ²	Diet × time
GPI						
0 h	3.76 ^b	4.39 ^{ab}	0.29	0.69	0.02	0.01
6 h	5.55 ^a	4.74 ^a				
API						
0 h	1.52	1.45	0.57	0.51	0.01	0.55
6 h	2.86	2.35				

^{a,b}Means within rows or columns with different superscripts differ ($P < 0.05$).

¹Control: basal diet with a forage-to-concentrate ratio of 50:50. The forage fraction in the GPI-control consisted of 50% alfalfa silage and 50% barley silage, whereas in the API-control it consisted of chopped alfalfa hay.

²Time of rumen fluid sampling immediately before and 6 h after feeding. Samples were collected 4 d after the start of SARA induction.

(MEGA ver. 4.0; Tamura et al., 2007). The fingerprints were also subjected to discriminant functional analysis (SPSS ver. 16.0; SPSS Inc., Chicago, IL) to evaluate how accurately rep-PCR fingerprints results were able to predict the source of *E. coli*. This was achieved by calculating the percentage of isolates from a given treatment that were correctly classified to the same treatment group, termed as rate of correct classification, and calculated using discriminant functional analysis with the “leave-one-out” classification method. Chi-squared analysis based on the Mantel Haenszel method (SAS, 2004) was performed to detect significant relationships between the treatments and *E. coli* clusters, phylogenotypes, and virulence genes. *Escherichia coli* abundance data were analyzed using the MIXED procedure of SAS (2004). The model statement included the effects of diet (control vs. SARA) and sampling time (0 vs. 6 h after feeding) as fixed variables. Cow served as the random variable. Statistical differences were considered significant at $P < 0.05$.

RESULTS

The abundance of *E. coli* isolated from the rumen fluid of GPI-fed cows ranged from 10^4 to 10^6 cfu/mL, which was approximately 3 logs higher than in the API-fed cows (Table 2). Rumen *E. coli* numbers were highest at 6 h after feeding in both GPI- and API-fed animals; however, no differences were found in *E. coli* counts between GPI-control and GPI-SARA or API-control and API-SARA (Table 2).

From the 129 isolates (GPI = 80 and API = 49; fewer colonies were obtained from the API group), 6 isolates belonging to the GPI group did not generate distinct banding patterns with rep-PCR, and were eliminated from further analysis. The DNA fingerprint patterns of the remaining 123 isolates were grouped

into 6 clusters. Clustering was affected by treatment ($P = 0.006$; Figure 1). Approximately 74% of isolates fell into 2 major clusters (clusters 1 and 4; Figure 1). Isolates from both controls and API-SARA were closely related irrespective of the diet, so that 46%, 70%, and 53% of isolates from GPI-control, API-control, and API-SARA, respectively, fell into cluster 4. In contrast, isolates from GPI-SARA (54%) grouped separately in cluster 1. The average rate of correct classification, which is defined as the percentage of isolates that can be correctly classified into their treatment group based on their fingerprint profile, was 59% of total isolates. The rate of correct classification of *E. coli* was 59.5% for GPI-control, 70% for API-control, 62.2% for GPI-SARA, and 36.8% for API-SARA (Table 3).

The difference in *E. coli* populations observed with rep-PCR was further confirmed by ABD typing. The ABD typing indicated a shift from an A-type *E. coli* population (23 control vs. 13 SARA) to a B1-type population (15 control vs. 23 SARA; $P = 0.04$) due to GPI-SARA (Table 4). In contrast, no significant changes existed in the ABD typing groups due to API-SARA. A significant relationship ($P = 0.04$) was found between *E. coli* clusters and ABD types (Figure 1). All B2-type *E. coli* were grouped in cluster 4, whereas 67% of D-type *E. coli* was grouped in clusters 3 and 4. The A- and B1-type *E. coli* were mostly distributed in clusters 1 and 4.

Of 25 *E. coli* virulence genes that we evaluated in this study (Table 1), 11 were not detected in any isolates (adhesins: *afaE*, *agg*, *bmaE*, *ompA*, *sfaD-E*; toxins: *ea*, *hlyA*, *vt1*, *vt2*; UPEC-specific protein: *usp*; pathogenicity island: *PAI*). Six virulence factors detected with low abundance in rumen *E. coli* (adhesins: *focG*, *pap*; toxins: *cnf*; capsule synthesis: *kpsMTII*, *neuC*; autotransporter: SPATE). Overall, a significant difference was found between API- and GPI-fed cows in 4 virulence

Table 3. Percentage (number) of rumen *Escherichia coli* from a given treatment that were correctly assigned to their group using discriminant analysis (leave-one-out classification method) of repetitive sequenced-based (rep)-PCR DNA fingerprints

		% (no.) of <i>E. coli</i> isolates classified as: ¹				
		GPI ²		API ³		
Challenge	Treatment	Control	SARA	Control	SARA	Total
GPI	Control	59.5 (22)	21.6 (8)	16.2 (6)	2.7 (1)	100 (37)
	SARA	24.3 (9)	62.2 (23)	5.4 (2)	8.1 (3)	100 (37)
API	Control	6.7 (2)	6.7 (2)	70.0 (21)	16.7 (5)	100 (30)
	SARA	5.3 (1)	26.3 (5)	31.6 (6)	36.8 (7)	100 (19)

¹Values in boldface indicate the rate of correct classification for each treatment. The average rate of correct classification was 59.3%.

²Grain-pellet feeding challenge.

³Alfalfa-pellet feeding challenge.

factors. The GPI-fed animals had greater *crl* (adhesin; $P < 0.01$), but fewer *agn43* (adhesin with autoaggregation ability; $P < 0.01$), *maIX* (pathogenicity island; $P < 0.01$), and *fliC*-positive isolates (flagellin; $P = 0.05$) compared with those of the API-fed group (Table 5). Even though the percentage of isolates carrying *agn43*, *maIX*, and *fliC* was higher in API-fed animals, the total number of rumen *E. coli* carrying these genes was about 3 logs higher in GPI-fed cows. Similarly, for *aidA*, *ompC*, and *cgsA* (all adhesins), the total rumen *E. coli* carrying them was 1,000 times higher in GPI-

than API-fed animals. No significant differences existed in the prevalence of virulence genes between control and SARA periods within API or GPI (Table 5). No significant relationship was detected between *E. coli* clusters or ABD types and presence of virulence genes.

DISCUSSION

The majority of production diseases in dairy cows occur around the transition and early to mid-lactation periods (Mulligan and Doherty, 2008). These include

Figure 1. The relatedness of *Escherichia coli* strains isolated from rumen fluid of dairy cows in grain pellet (GPI)- and alfalfa pellet (API)-control, and GPI- and API-SARA feeding challenges. Dendrograms were constructed from repetitive sequence-based (rep)-PCR DNA fingerprints of *E. coli* isolates using maximum composite likelihood and unweighted pair-groups method with arithmetic mean clustering methods. Values represent the number of *E. coli* isolates in each cluster/treatment group, and the distribution of A, B1, and B2+D types in each cluster.

Table 4. Percentage (number) of rumen *Escherichia coli* assigned to A, B1, and B2+D groups in grain pellet (GPI)- and alfalfa pellet (API)-control, and GPI- and API-SARA feeding challenges; and statistical relationships between groups A, B, D, and treatments

Item	% (no.) of <i>E. coli</i> isolates with phylotype during:				<i>P</i> -value of comparison	
	GPI		API		GPI ABD group	API ABD group
	Control	SARA	Control	SARA		
<i>E. coli</i> phylotype						
A	57.5 (23)	32.5 (13)	50.0 (15)	47.4 (9)		
B1	37.5 (15)	57.5 (23)	43.3 (13)	36.8 (7)		
B2+D	5.0 (2)	10.0 (4)	6.7 (2)	15.8 (3)		
Total	100 (40)	100 (40)	100 (30)	100 (19)		
ABD group						
B1 vs. A ¹					0.04	0.99
B2+D vs. A+B1 ²					0.61	0.06

¹Abundance of B1 vs. A in SARA compared with that in control.

²Abundance of B2+D vs. A+B1 in SARA compared with control.

metabolic diseases (e.g., acidosis, ketosis, and fatty liver) as well as mastitis (Mulligan and Doherty, 2008). Of these diseases, only mastitis has been associated with pathogenic microorganisms (Pyörälä, 2008), and the microbiology of acidosis has been considered only in relation to the imbalance of lactate-producing and consuming rumen bacteria (Nagaraja and Titgemeyer, 2007). Less information is available on the microbial etiology of SARA (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2008). We recently published a comprehensive molecular microbial analysis of the rumen during SARA and demonstrated significant changes compared with controls (Khafipour et al., 2009c). One

of the most interesting changes was related to *E. coli*, which we hypothesized may play a role in immune activation during SARA. The current study demonstrated that the numbers of *E. coli* in GPI- SARA were higher than API-SARA (Table 2) and that they carried virulence factors (Table 5) that could partly explain the inflammatory response associated with GPI-SARA.

Even though the numbers of *E. coli* in API-SARA cows were lower than those from GPI-SARA (Table 2), the concentration of free LPS in the rumen fluid was higher in API-SARA (Khafipour et al., 2009a, b). Because LPS is readily released from intact cells during their logarithmic phase of growth (Hurley, 1995),

Table 5. Distribution of virulence genes¹ present in rumen *Escherichia coli* isolated from grain pellet (GPI)- and alfalfa pellet (API)-control, and GPI- and API-SARA feeding challenges; and statistical relationships between virulence factors and challenges

% of <i>E. coli</i> isolates (cfu/mL of rumen fluid) with virulence genes during:					
Virulence genes	GPI		API		<i>P</i> -value of API vs. GPI ² challenge
	Control	SARA	Control	SARA	
<i>agn43</i>	28.9 (13,059)	15.0 (5,509)	79.4 (123)	78.9 (63)	<0.01
<i>aidA</i>	55.3 (24,988)	47.5 (17,446)	55.9 (87)	31.6 (25)	NS
<i>cgsA</i>	100.0 (45,186)	100.0 (36,728)	94.1 (146)	100.0 (79)	NS
<i>cnf</i>	0.0 (0)	0.0 (0)	8.8 (14)	0.0 (0)	NS
<i>crl</i>	100.0 (45,186)	100.0 (36,728)	73.5 (114)	94.74 (75)	<0.01
<i>fliC</i>	71.1 (32,127)	70.0 (25,710)	94.1 (146)	89.5 (71)	0.05
<i>focG</i>	0.0 (0)	0.0 (0)	5.9 (9)	0.0 (0)	NS
<i>kpsMT II</i>	2.6 (1,175)	0.0 (0)	2.9 (4)	5.3 (4)	NS
<i>malX</i>	47.4 (21,418)	35.0 (12,855)	93.9 (145)	52.6 (42)	<0.01
<i>neuC</i>	13.2 (5,964)	5.0 (1,836)	14.7 (23)	0.0 (0)	NS
<i>ompC</i>	92.1 (41,616)	92.5 (33,974)	100.0 (155)	100 (79)	NS
<i>pap</i>	2.6 (1,175)	0.0 (0)	0.0 (0)	0.0 (0)	NS
<i>SPATE</i>	5.3 (2,395)	7.5 (2,755)	11.8 (18)	0.0 (0)	NS

¹Virulence genes *afaE*, *agg*, *bmaE*, *eae*, *hlyA*, *ompA*, *PAII*, *sfaD-E*, *usp*, *vt1*, and *vt2* were not detected in any isolate. See Table 1 for definition of each virulence gene.

²Abundance of virulence gene in the API compared with the GPI. Statistical analyses are based on the percentage of *E. coli* isolates.

this suggests that a large proportion of free rumen LPS is produced by non-*E. coli* gram-negative bacteria. However, the toxicity of LPS from other rumen gram-negative bacteria is far less than that of *E. coli* LPS (Brigham, 1994; Hurley, 1995). For example, *Fibrobacter succinogenes* and *Bacteroides* spp. in these cows were more abundant (Khafipour et al., 2009c) but it has been demonstrated that the toxicity of their LPS is low (Kaspar 1976; Nagaraja et al., 1979a, b). The limulus amebocyte lysate test that we used measures total LPS but does not assess differences in the toxicity of LPS molecules (Hurley, 1995). Thus, even if *E. coli* numbers are relatively low compared with those of other gram-negative bacteria, their contribution to the pathophysiology of SARA is likely to be greater because they have greater virulence potential than *Bacteroides* spp. and *F. succinogenes* (Kaspar 1976; Nagaraja et al., 1979a, b).

Feeding alfalfa pellets or grain caused significant reductions in rumen pH and increased osmolarity (Khafipour et al., 2009a, b). With both diets, parakeratosis of the rumen epithelium may result (Hinders and Owen, 1965; Tamate et al., 1978; Steele et al., 2009). Hinders and Owen (1965) fed alfalfa pellets or long hay, and observed that animals on the pelleted alfalfa diet had reddening and slight hypertrophy of the rumen papillae within 2 wk, which became rough with multiple localized adhesions after wk 3. In a case report, Steele et al. (2009) transitioned a dairy cow from a high-forage diet to a high-concentrate diet (79%) for 1 wk and observed extensive sloughing of the stratum corneum of rumen papillae and abnormally high numbers of nondifferentiated keratinocytes, which is a hallmark of parakeratosis.

In a healthy rumen, keratinized cells cover the epithelium, but during parakeratosis the rate of cellular differentiation and the movement of cells toward the surface are increased so that the time necessary for complete keratinization is reduced to below a minimum, and thus, an abnormally high proportion of immature nonkeratinized cells reach the surface of the epithelium (Dirksen et al., 1984). Gálfi et al. (1998) cultured *E. coli* in the presence of keratinized and nonkeratinized cells and demonstrated that nonkeratinized cells bound more *E. coli* than did keratinized cells. Semjén and Gálfi (1990) demonstrated that *E. coli* preferentially adhered to cultured nonkeratinized rumen epithelial cells with type I fimbriae and caused mannose sensitive hemagglutination. There appears to be a general reduction of attachment sites for *E. coli* when epithelia are keratinized (Semjén et al., 1982). On the other hand, lower microbial diversity on parakeratotic epithelia (Steele et al., 2009) potentially increases the opportunity for *E. coli* attachment. The change in microbial popula-

tion competition at the epithelia likely also changes the composition of *E. coli* to a population that is able to take advantage of a SARA environment (Table 4, Figure 1).

In our experiments, *E. coli* were isolated from the rumen of animals on either API or GPI diets with low pH (250–550 min below pH 5.6) and high osmolarity (330 mOsm/kg) (Khafipour et al., 2009a, b). Although the rumen epithelia in these animals were not examined, we can assume, based on the literature (Hinders and Owen, 1965; Tamate et al., 1978; Steele et al., 2009), that parakeratosis was present. Thus, in both diets, nonkeratinized rumen epithelia would have been available for binding to *E. coli*, but the number of rumen *E. coli* in the GPI-SARA was approximately 1,000 times higher than in the API-SARA (Table 2), which would have increased the opportunity of the bacterium to take advantage of binding sites. The availability of readily fermentable carbohydrate results in an increase in the abundance of *E. coli* in the rumen (Russell et al., 2000; Krause et al., 2003), and the inclusion of forage reduces *E. coli* abundance (Callaway et al., 2009). We concluded that low rumen pH and high osmolarity alone, although damaging to the rumen epithelium, are not enough to cause immune activation during SARA. An energy source, such as nonstructural carbohydrate that can be used by pathogenic bacteria like *E. coli* is also required.

For *E. coli* to exert a pathogenic effect, it would likely have to possess a combination of virulence factors that help the bacterium attach to nonkeratinized epithelial cells, invade the host, avoid or disrupt host defense mechanisms, injure the host tissue, and stimulate an inflammatory response (Johnson and Stell, 2000). Our results showed that *E. coli* from either GPI- or API-SARA were less likely to possess genes encoding for enterotoxins (*cnf*, *eae*, *hlyA*, *vt1*, *vt2*; Table 5). In contrast, genes encoding for adhesins were more abundant but were not found exclusively in the isolates from GPI and were also present in API isolates, indicating that these potential pathogens are always present but it is the availability of readily fermentable carbohydrate from grain that results in an increase in abundance of the bacterium and, consequently, its association with disease.

A significant ($P < 0.01$) association existed between *E. coli* possessing curli fibers (*crl*) and GPI group (Table 5). Curli fibers are protein components of a complex extracellular matrix produced by *E. coli* and were first discovered in *E. coli* strains that caused bovine mastitis (Olsén et al., 1989). Curli fibers enable adhesion to surfaces, cell aggregation, and biofilm formation. They may also induce host cell adhesion and invasion, and upregulate the host inflammatory response. It is, thus,

plausible that curli fiber-associated *E. coli* reside in the rumen and under appropriate physiological conditions (e.g., parakeratosis of rumen wall) cause inflammation of the rumen epithelium. This hypothesis supports our previous observation that mediators of inflammatory response were increased only due to GPI-SARA (Khafipour et al., 2009a, b).

The higher numbers of *E. coli* associated with GPI-SARA also had higher pathogenic potential because a larger population of the bacterium possessed virulence factors *agn43*, *aidA*, *cgsA*, *fliC*, *ompC*, and *malX* (Table 5). A large population of *E. coli* is essential for the expression of its virulence genes because the gene expression is regulated in response to cell density by quorum sensing (Ng and Bassler, 2009). Quorum sensing, or cell-to-cell communication, is a method by which *E. coli* coordinates its population behavior in response to its own population density or that of other members of the microbiome, as well as the activity of the host (Anand and Griffiths, 2003). This allows *E. coli* to act in unison, and to appropriately time the expression of virulence factors when the bacteria are in sufficient numbers to overwhelm the host defense (Anand and Griffiths, 2003).

Among the above-mentioned virulence genes, *agn43* and *aidA* encode antigen 43 and AIDA-I proteins, respectively. Like curli fibers, these proteins facilitate *E. coli* adhesion to the epithelial surface (Le Bouguenec, 2005). Antigen 43 and AIDA-I also have a major role in aggregation and biofilm formation of the *E. coli* (Henderson et al., 2004; Hatt and Rather, 2008), an ability that increases bacterial resistance to host defense mechanisms. In addition, *cgsA* is a key virulence gene that encodes the curlin subunit of the curli fibers and its expression is regulated by *crl* (Maurer et al., 1998). These results suggest that rumen *E. coli* has evolved to produce several adhesins that are essential for the bacterium pathogenesis.

In conclusion, we have identified genes in rumen *E. coli* for curli fiber synthesis that are potent virulence factors. Furthermore, we have demonstrated that under low rumen pH conditions induced by a high-grain diet, there is a burst in the number of *E. coli* with virulence genes that can take advantage of these rumen conditions to trigger an inflammatory response.

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