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Lipopolysaccharide Stimulates the Growth of Bacteria That Contribute to Ruminal Acidosis

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ABSTRACT Lipopolysaccharide (LPS) has been reported to contribute to a ruminal acidosis of cattle by affecting ruminal bacteria. The goal of this study was to determine how LPS affects the growth of pure cultures of ruminal bacteria, including those that contribute to ruminal acidosis. We found that dosing LPS (200,000 EU) increased the maximum specific growth rates of four ruminal bacterial species (*Streptococcus bovis* JB1, *Succinivibrio dextrinosolvens* 24, *Lactobacillus ruminis* RF1, and *Selenomonas ruminantium* HD4). Interestingly, all the species ferment sugars and produce lactate, contributing to acidosis. Species that consume lactate or ferment fiber were not affected by LPS. We found that *S. bovis* JB1 failed to grow in LPS as the carbon source in the media; growth of *S. bovis* JB1 was increased by LPS when glucose was present. Growth of *Megasphaera elsdenii* T81, which consumes lactate, was not different between the detoxified (lipid A delipidated) and regular LPS. However, the maximum specific growth rate of *S. bovis* JB1 was greater in regular LPS than detoxified LPS. Mixed bacteria from a dual-flow continuous culture system were collected to determine changes of metabolic capabilities of bacteria by LPS, and genes associated with LPS biosynthesis were increased by LPS. In summary, LPS was not toxic to bacteria, and lipid A of LPS stimulated the growth of lactate-producing bacteria. Our results indicate that LPS not only is increased during acidosis but also may contribute to ruminal acidosis development by increasing the growth of lactic acid-producing bacteria.

IMPORTANCE Gram-negative bacteria contain lipopolysaccharide (LPS) coating their thin peptidoglycan cell wall. The presence of LPS has been suggested to be associated with a metabolic disorder of cattle—ruminal acidosis—through affecting ruminal bacteria. Ruminal acidosis could reduce feed intake and milk production and increase the incidence of diarrhea, milk fat depression, liver abscesses, and laminitis. However, how LPS affects bacteria associated with ruminal acidosis has not been studied. In this study, we investigated how LPS affects the growth of ruminal bacteria by pure cultures, including those that contribute to acidosis, and the functional genes of ruminal bacteria. Thus, this work serves to further our understanding of the roles of LPS in the pathogenesis of ruminal acidosis, as well as providing information that may be useful for the prevention of ruminal acidosis and reducetion of economic losses for farmers.

KEYWORDS LPS, dairy cow, Gram-negative bacteria, ruminal bacteria

Gram-negative bacteria contain lipopolysaccharide (LPS) coating their thin peptidoglycan cell wall, which is important for the structural and functional integrity of the bacteria (1). Lipopolysaccharides from all Gram-negative bacteria share essentially the same structure: an O-polysaccharide chain, an R core, and a lipid A moiety; the lipid A moiety, covalently linked to the inner core, carries the endotoxic activity of LPS (2, 3).

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The presence of LPS in the plasma has been correlated to multiple diseases, including sepsis and septic shock but also more recently to obesity and its associated metabolic disorders, in human beings (1). The presence of LPS also has been reported to contribute to a significant metabolic disorders in dairy cattle: subacute ruminal acidosis (SARA) (4). Subacute ruminal acidosis has been associated with reduced feed intake and milk production, diarrhea, milk fat depression, liver abscesses, and laminitis (5–7). The adverse effects of SARA are very costly and can affect animal health, especially for high-producing dairy cows (5).

In general, ruminal bacteria are predominantly Gram negative (8), and these bacteria are the major source of LPS in the rumen. Bacteria death and lysis are normal processes; LPS is thus normally present in ruminal fluid. However, the LPS concentration is much higher in grain-fed than in forage-fed cattle (9, 10), especially in cows with SARA, in which the LPS concentration ranges from 4- to 16-fold higher than in cows without SARA (11–13). Therefore, LPS has been suspected to contribute to the pathogenesis of SARA (4). Meanwhile, our previous study has reported that LPS dosing (200,000 endotoxin units [EU]) changed the ruminal bacterial population and stimulated the Gram-negative bacteria related to starch digestion, which may, in turn, develop into SARA (14).

However, how LPS alters the struture of ruminal bacteria and stimulates bacteria associated with ruminal acidosis has not been well studied. Therefore, the goal of this study was first to determine how LPS affects the growth of pure cultures of ruminal bacteria, including those that contribute to SARA, and then to evaluate how LPS affects the functional genes of mixed ruminal bacteria by shotgun sequencing. We expected that the current study would facilitate our understanding of the role of LPS on pathogenesis of SARA, as well as provide information that may be useful for the prevention of ruminal acidosis. We hypothesized that (i) dosing of LPS would affect Gram-negative bacteria that use starch as the substrate, (ii) LPS would replace glucose as a carbon source for growth of ruminal lactate-producing bacteria, (iii) LPS would be a growth factor for lactate-producing bacteria and toxic to non-starch-utilizing bacteria, and (iv) LPS would change functional genes of ruminal bacteria.

RESULTS

Effects of LPS on bacterial growth and fermentation end products. In order to evaluate if LPS could only stimulate Gram-negative bacteria that utilize starch, we applied 200,000 EU of regular LPS to eight different ruminal bacterial species in pure culture. We found that the maximum specific growth rates of lactate-producing bacteria (*Succinivibrio dextrinosolvens* 24, *Selenomonas ruminantium* HD4, *Lactobacillus ruminis* RF1, and *Streptococcus bovis* JB1) were increased by regular LPS (R-LPS) dosing (200,000 EU) regardless of Gram reaction (Table 1). The concentration of LPS was chosen based on previous studies in which the ruminal concentration of LPS was measured in cows with SARA (11–13). The maximum specific growth rate of *S. bovis* JB1 was increased by 5.88%, that of *Se. ruminantium* HD4 by 7.83%, that of *Su. dextrinosolvens* 24 by 8.21%, and that of *L. ruminis* RF1 by 29.1% by LPS dosing (Table 1). The lag phases of *Se. ruminantium* HD4 and *Su. dextrinosolvens* 24 were reduced by LPS dosing (Table 1). However, dosing of R-LPS had no effects on the maximum specific growth rate or lag phase of fiber-utilizing bacteria (*R. albus* 7, *Ruminococcus flavefaciens* FD-1, and *Fibrobacter succinogenes* S85 (Table 1) or lactate-utilizing bacteria (*Megasphaera elsdenii* T81) (Table 1). Therefore, the results showed that LPS stimulated the growth of bacteria that ferment starch and produce lactate regardless of Gram reaction; however, species that consume lactate or ferment fiber were not affected by LPS.

In order to determine the mechanism by which LPS stimulates growth of amylolytic lactic acid bacteria, we grew *S. bovis* JB1 in defined mediaum without a carbon source and added regular LPS as the only carbon source. We found *S. bovis* JB1 did not grow on it (Fig. 1), indicating that LPS cannot be utilized as the main carbon source for bacterial growth of *S. bovis* JB1. However, when glucose was present, *Streptococcus bovis* JB1 grew on it, and the maximum specific growth rate of *S. bovis* JB1 was increased by 15% compared to that of the control (Table 2), which indicates that the

TABLE 1 Effects of regular lipopolysaccharide on lag phase and the maximum specific growth rate of bacteria based on the prediction from logistic function[a]

Bacterium and parameter	Value with treatment[b]		SEM	*P* value
	Control	R-LPS		
Starch-utilizing bacteria				
Selenomonas ruminantium HD4				
Lag, min	243	234	5.23	0.02
μmax, h^{-1}	0.35	0.38	0.06	<0.01
Succinivibrio dextrinosolvens 24				
Lag, min	220	212	5.17	0.03
μmax, h^{-1}	0.11	0.12	0.00	<0.01
Lactobacillus ruminis RF1				
Lag, min	158	173	35.7	0.23
μmax, h^{-1}	0.07	0.09	0.02	<0.01
Streptococcus bovis JB1				
Lag, min	165	152	14.3	0.24
μmax, h^{-1}	1.02	1.08	0.06	0.04
Fiber-utilizing bacteria				
Ruminococcus albus 7				
Lag, min	402	396	26.2	0.54
μmax, h^{-1}	0.40	0.41	0.14	0.21
Ruminococcus flavefaciens FD-1				
Lag, min	567	563	58.9	0.78
μmax, h^{-1}	0.18	0.18	0.01	0.98
Fibrobacter succinogenes S85				
Lag, min	446	449	54.3	0.43
μmax, h^{-1}	0.20	0.20	0.01	1.00
Lactate-utilizing bacterium				
Megasphaera elsdenii T81				
Lag, min	253	238	42.3	0.54
μmax, h^{-1}	0.14	0.14	0.00	0.76

[a]Lag, lag phase; μmax, maximum specific growth rate.
[b]Control, control group (LPS-free anaerobic water); R-LPS, regular LPS (200,000 EU).

stimulating effects of LPS on ruminal bacteria occurs if other carbon sources are available. To validate this, glucose was then added to the Balch tubes in which *S. bovis* JB1 did not grow. We found that *S. bovis* JB1 regrew and that the maximum specific growth rate of *S. bovis* JB1 was increased by 15% (Fig. 1; Table 2) and the lag phase was reduced by 40 min by LPS dosing compared to those of the control (Table 2). Therefore,

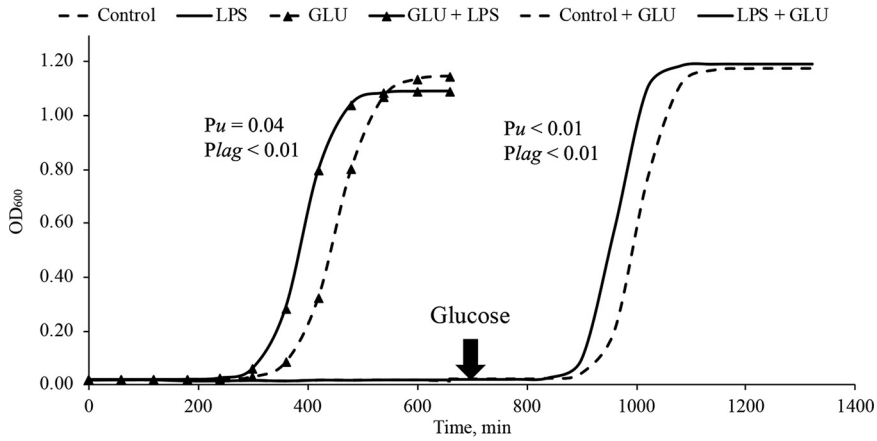

FIG 1 Effects of replacing glucose with lipopolysaccharide as a carbon source on the growth of *Streptococcus bovis* JB1. Control, control group (LPS-free anaerobic water); LPS, regular LPS (200,000 EU) as a carbon source; GLU, glucose as a carbon source; GLU + LPS, glucose plus LPS (200,000 EU); control + GLU, control group plus glucose; LPS + GLU, regular LPS (200,000 EU) plus glucose.

TABLE 2 Effects of regular lipopolysaccharide as a carbon source on the lag phase and the maximum specific growth rate of *Streptococcus bovis* JB1 based on the logistic function prediction

Parameter	Value with treatment[a]				SEM	*P* value
	Control[b]	R-LPS[b]	GLU + control[c]	GLU + R-LPS[c]		
Without glucose added						
Lag, min	—	—	387	318	6.83	<0.01
μmax, h^{-1}	—	—	0.50	0.58	0.10	0.04
With glucose added						
Lag, min	292	256	—	—	5.72	<0.01
μmax, h^{-1}	0.66	0.76	—	—	0.18	<0.01

[a]Control, control group (LPS-free anaerobic water); R-LPS, regular LPS (200,000 EU) as a carbon source; GLU, glucose as a carbon source; GLU + R-LPS, glucose plus R-LPS (200,000 EU).
[b]Results were obtained after adding glucose. —, not applicable (no growth).
[c]—, data not shown.

this confirmed that the stimulating effects of LPS on ruminal bacteria required other carbon sources to be present.

The LPS mainly consists of two components: polysaccharide and fatty acid. In order to evaluate which part would be the stimulator, the same concentrations of regular and detoxified LPS (D-LPS; lipid A delipidated) were applied to evaluate their effects on the growth of *S. bovis* JB1. We found that the maximum specific growth rate of *S. bovis* was increased by 7.5% in D-LPS (lipid A delipidated) and increased by 15% in R-LPS compared to that of the control (Table 3). The lag phase of *S. bovis* JB1 was not affected by D-LPS dosing, while it was reduced by 42 min by R-LPS dosing compared to that of the control (Table 3), indicating that lipid A may be the stimulator.

In order to evaluate if LPS was toxic to ruminal bacteria, the same concentration of regular and detoxified LPS (D-LPS; lipid A delipidated) were applied to evaluate their effects on the growth of *M. elsdenii* T81. We found that the maximum specific growth rate and lag phase of *M. elsdenii* T81 were not different among the control, detoxified, and regular LPS dosing (Table 3). Meanwhile, the growth rate of *S. bovis* JB1was greater in R-LPS and D-LPS. Therefore, we can conclude that LPS is not toxic to *S. bovis* JB1, the main lactic acid-producing bacteria during SARA.

However, we found that concentrations of fermentation end products (volatile fatty acids [VFA] and NH_3-N) were not affected by treatments for all the tested bacteria, and concentrations of L-lactate, D-lactate, and total lactate were not affected by treatment for all tested bacteria as well (Tables 4 to 7).

Ruminal microbiome functional changes by LPS dosing. In order to evaluate the effects of LPS on metabolic capabilities of ruminal bacteria, we dosed 200,000 EU of R-LPS in a dual-flow continuous culture system and then mixed ruminal bacteria which were collected to perform shotgun sequencing. We found that ruminal microbiome

TABLE 3 Effects of detoxified and regular lipopolysaccharide on lag phases and the maximum specific growth rates of *Megasphaera elsdenii* T81 and *Streptococcus bovis* JB1 based on the prediction from logistic function

Bacterium and parameter	Value with treatment[a]			SEM	*P* value
	Control	D-LPS	R-LPS		
Megasphaera elsdenii T81					
Lag, min	181	172	158	24.1	0.15
μmax, h^{-1}	0.12	0.12	0.12	0.00	0.21
Streptococcus bovis JB1					
Lag, min	293 A	285 A	251 B	6.07	<0.01
μmax, h^{-1}	0.67 C	0.72 B	0.77 A	0.03	<0.01

[a]Control, control group (LPS-free anaerobic water); D-LPS, detoxified LPS (200,000 EU); R-LPS, regular LPS (200,000 EU). Least-squares means within the same row with different letters are significantly different ($P < 0.05$).

TABLE 4 Effects of regular lipopolysaccharide on ammonia nitrogen, VFA, and lactate concentrations in lactate-producing bacteria

Bacterium and parameter	Value (mM) with treatment[a]		SEM	*P* value
	Control	R-LPS		
Selenomonas ruminantium HD4				
NH_3-N	3.45	3.47	0.35	0.90
Acetate	10.9	11.6	3.73	0.60
Propionate	4.85	5.08	0.49	0.23
Valerate	2.94	2.90	0.56	0.79
D-Lactate	5.07	4.96	0.09	0.42
L-Lactate	0.33	0.34	0.04	0.86
DL-Lactate	5.40	5.30	0.08	0.41
Succinivibrio dextrinosolvens 24				
NH_3-N	3.94	4.07	0.34	0.51
Acetate	27.9	28.3	0.04	0.22
Propionate	5.99	6.01	0.47	0.60
Butyrate	3.32	3.34	0.02	0.65
D-Lactate	4.50	4.43	0.13	0.44
L-Lactate	—	—	—	—
DL-Lactate	4.50	4.43	0.13	0.44
Lactobacillus ruminis RF1				
NH_3-N	4.08	4.27	0.50	0.37
Acetate	41.3	32.5	3.19	0.09
Propionate	13.3	10.6	0.92	0.17
Butyrate	6.99	5.63	0.54	0.19
D-Lactate	0.99	1.00	0.02	0.42
L-Lactate	7.50	7.20	0.51	0.35
DL-Lactate	8.50	8.22	0.56	0.41
Streptococcus bovis JB1 with PC+VFA				
NH_3-N	4.05	3.83	0.14	0.30
Acetate	59.7	65.2	4.23	0.35
Valerate	0.91	0.85	0.05	0.34
D-Lactate	0.14	0.15	0.06	0.85
L-Lactate	6.81	7.13	0.68	0.47
DL-Lactate	6.96	7.28	0.64	0.45

[a]Control, control group (LPS-free anaerobic water); LPS, regular LPS (200,000 EU). Lactate concentrations are millimolar. —, not applicable.

functions were different between the control and LPS dosing (Fig. 2). The relative abundances of sequence reads of 35 functional genes and 86 functional genes were affected by LPS dosing based on KEGG data set and SEED data set, respectively (see Fig. S1 and S2 in the supplemental material). LPS dosing showed a greater abundance of sequences of functional genes related to the pentose phosphate pathway (PPP) (Fig. 3A), Entner-Doudoroff pathway (EDP) (Fig. 3B), and 3-deoxy-D-manno-octulosonic acid (2-keto-3-deoxyoctonate [KDO_2])–lipid A biosynthesis (Fig. 3C) than the control. The proportions of sequences associated with transaldolase (Fig. 4A), ribose 5-phosphate isomerase B (*rpiB*) (Fig. 4B), and D-glycero-D-manno-heptose 1,7-bisphosphate phosphatase (*gmhB*) (Fig. 4C) were increased by LPS dosing, while the proportion of sequences associated with LPS export system ATP-binding protein (*lptB*) (Fig. 4D) was decreased by LPS dosing compared to that of the control. Meanwhile, the abundances of sequence of functional genes related to 2-dehydro-3-deoxygluconokinase (*kdgK*) (Fig. 4E) and deoxy-D-gluconate 3-dehydrogenase (*kduD*) (Fig. 4F) were increased by LPS dosing as well. Sequence abundances of functional genes associated with *methyl*-coenzyme M reductase beta subunit (*mcrB*) (Fig. 5A) and sequence abundance of *Methanococcoides burtonii* (Fig. 5B) were decreased by LPS dosing.

DISCUSSION

Based on our previous study, LPS dosing (200,000 EU) stimulated the Gram-negative bacteria related to starch digestion (*Succinimonas*, *Anaeroplasma*, *Succinivibrio*, *Succini-*

TABLE 5 Effects of regular lipopolysaccharide on ammonia nitrogen, VFA, and lactate concentrations in fiber- and lactate-utilizing bacteria

Bacterium and parameter	Value with treatment[a]		SEM	*P* value
	Control	R-LPS		
Ruminococcus albus 7				
NH_3-N	2.28	2.04	0.35	0.26
Acetate	26.5	25.8	0.35	0.24
Propionate	5.47	5.18	0.60	0.75
Butyrate	2.88	2.66	0.91	0.69
Ruminococcus flavefaciens FD-1				
NH_3-N	1.97	1.80	0.13	0.13
Acetate	31.6	31.3	1.08	0.64
Propionate	6.12	6.11	0.21	0.96
Butyrate	3.33	3.33	0.12	1.00
Fibrobacter succinogenes S85				
NH_3-N	1.69	1.79	0.31	0.59
Acetate	26.6	27.3	1.23	0.50
Propionate	5.91	6.08	0.26	0.51
Butyrate	3.28	3.34	0.14	0.54
Megasphaera elsdenii T81				
NH_3-N	6.18	6.24	0.97	0.92
Acetate	9.16	10.0	1.47	0.69
Butyrate	6.25	6.57	0.71	0.73
Valerate	3.02	2.80	0.39	0.71
D-Lactate	0.10	0.16	0.03	0.28
L-Lactate	0.07	0.15	0.18	0.30
DL-Lactate	0.15	0.29	0.08	0.28

[a]Control, control group (LPS-free anaerobic water); LPS, regular LPS (200,000 EU). Lactate concentrations are millimolar.

clasticum, and *Ruminobacter*) (14). Therefore, we hypothesized that LPS dosing would only affect Gram-negative bacteria that mainly used starch or another soluble carbohydrate as their main substrates. From the current study, we found that LPS dosing increased the maximum specific growth rates while decreasing the lag phases of *Su. dextrinosolvens* 24 and *Se. ruminantium* HD4, Gram-negative bacteria that mainly use starch as a substrate (15, 16). However, dosing LPS also increased the maximum specific

TABLE 6 Effects of regular lipopolysaccharide as the carbon source on ammonia nitrogen, VFA, and lactate concentrations in *Streptococcus bovis* JB1 with defined media

Parameter	Value with treatment[a]				SEM	*P* value
	Control[b]	R-LPS[b]	GLU + Control[c]	GLU + R-LPS[c]		
Without glucose added						
NH_3-N	—	—	2.10	2.25	0.14	0.40
Acetate	—	—	37.4	38.1	2.16	0.76
Valerate	—	—	1.51	1.46	0.23	0.18
D-Lactate	—	—	0.04	0.05	0.01	0.77
L-Lactate	—	—	8.83	8.88	0.08	0.79
DL-Lactate	—	—	8.85	8.90	0.09	0.77
With glucose added						
NH_3-N	2.07	2.40	—	—	0.18	0.26
Acetate	32.4	33.2	—	—	1.35	0.66
Valerate	1.97	1.53	—	—	0.34	0.21
D-Lactate	0.04	0.05	—	—	0.01	0.69
L-Lactate	8.77	8.83	—	—	0.11	0.71
DL-Lactate	8.78	8.85	—	—	0.12	0.71

[a]Control, control group (LPS-free anaerobic water); R-LPS, regular LPS (200,000 EU) as a carbon source; GLU, glucose as a carbon source; GLU + R-LPS, glucose plus R-LPS (200,000 EU). Lactate concentrations are millimolar.
[b]Results were obtained after adding glucose. —, not applicable (no growth).
[c]—, data not shown.

TABLE 7 Effects of detoxified and regular lipopolysaccharide on ammonia nitrogen, VFA, and lactate concentrations in *Megasphaera elsdenii* T81 and *Streptococcus bovis* JB1 with defined media

Bacterium and parameter	Value with treatment[a]			SEM	*P* value
	Control	D-LPS	R-LPS		
Megasphaera elsdenii T81					
NH_3-N	5.82	5.75	5.48	0.29	0.63
Acetate	12.5	13.3	12.2	1.46	0.83
Butyrate	7.87	8.98	7.67	0.88	0.56
Valerate	2.37	2.24	2.35	0.19	0.84
D-Lactate	0.12	0.14	0.13	0.01	0.55
L-Lactate	0.06	0.06	0.05	0.01	0.53
DL-Lactate	0.18	0.20	0.18	0.02	0.55
Streptococcus bovis JB1					
NH_3-N	2.75	2.54	2.48	0.11	0.13
Acetate	44.8	47.2	46.7	4.83	0.41
Valerate	1.32	1.25	1.34	0.15	0.41
D-Lactate	0.00	0.01	0.01	0.01	0.57
L-Lactate	8.80	8.72	8.74	0.08	0.75
DL-Lactate	8.82	8.74	8.76	0.08	0.79

[a]Control, control group (LPS-free anaerobic water); D-LPS, detoxified LPS (200,000 EU); R-LPS, regular LPS (200,000 EU). Lactate cconcentrations are millimolar.

growth rates of *S. bovis* JB1 and *L. ruminis* RF1, which are abundant species in the rumens of animals adapted to high-grain diets (17) and in the rumens of animals with acidosis (18). Even though they are also starch utilizers and lactate producers, both are Gram-positive bacteria. Therefore, the current results were against our first hypothesis that LPS dosing would only affect Gram-negative bacteria that mainly used starch or another soluble carbohydrate as a substrate. Actually, dosing of LPS could stimulate lactate-producing bacteria regardless of their Gram reactions.

Ruminal bacteria that rapidly ferment starch or soluble sugars and contribute to rapid accumulations of DL-lactic acid and VFA include *S. dextrinosolvens*, *Se. ruminantium*, *S. bovis*, and *Lactobacillus* spp., which are considered to play important roles in ruminal acidosis (18). Therefore, the stimulating effect of LPS on the growth of these lactate-producing bacteria indicates that LPS not only is produced during acidosis but also may contribute to ruminal acidosis development.

Due to its central role in the prevention of acid accumulation in the rumen, *Megasphaera elsdenii* (Gram negative) is also predominant in the rumens of animals with ruminal acidosis (19). However, dosing of LPS had no effects on the maximum specific growth rate or lag phase of *M. elsdenii* T81, as well as its fermentation end products. Meanwhile, we sought to evaluate if LPS dosing would affect other bacteria; therefore, three main fiber-utilizing bacteria (*R. albus* 7, *R. flavefaciens* FD-1, and *F. succinogenes* S85) that use cellobiose as their substrates were tested, and we found that LPS dosing had no effects on their maximum specific growth rates, lag phases, or fermentation end products. Therefore, dosing of LPS mainly stimulated lactate-producing bacteria and not the fiber-utilizing (*R. albus* 7, *R. flavefaciens* FD-1, and *F. succinogenes* S85) or lactate-utilizing (*M. elsdenii* T81) bacteria based on the current study.

Next, we wished to investigate the potential mechanism of how LPS stimulates the growth of these lactate-producing bacteria. LPS is an amphipathic molecule that contains fatty acyl chains attached to a polysaccharide containing up to 200 sugars (20). Therefore, we thought that LPS may be catabolized by the enzymes secreted by bacteria and used as the carbon source for their growth. *Streptococcus bovis* JB1 with the defined medium was used to test this hypothesis. We found that when LPS replaced glucose as the carbon source in the defined medium, *S. bovis* JB1 did not grow on it. However, when the glucose was added into the LPS dosing medium, *Streptococcus bovis* JB1 started to grow and the maximum specific growth rate was increased.

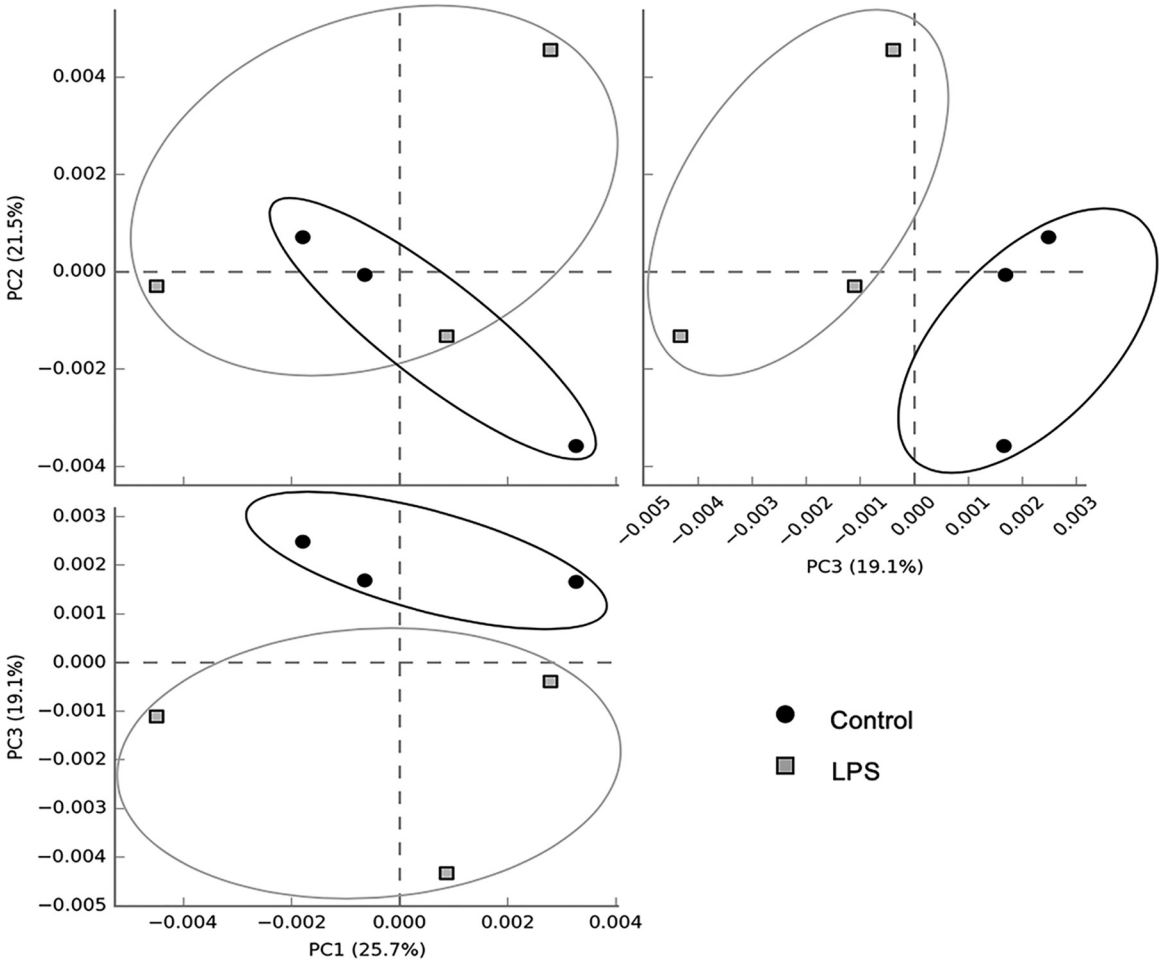

FIG 2 Principal-coordinate analysis plots (PCA) performed on microbial functional similarities between control and lipopolysaccharide dosing in a dual-flow continuous culture system based on the KEGG database.

Meanwhile, the stimulating effect of LPS on the growth of *S. bovis* JB1 was present regardless of time of glucose inclusion. LPS dosing increased the maximum specific growth rate about 15% before and after glucose addition. Therefore, LPS could not replace glucose as the carbon source for *S. bovis* JB1 growth, and the stimulating effect of LPS on the growth of lactate-producing bacteria can be observed when there is

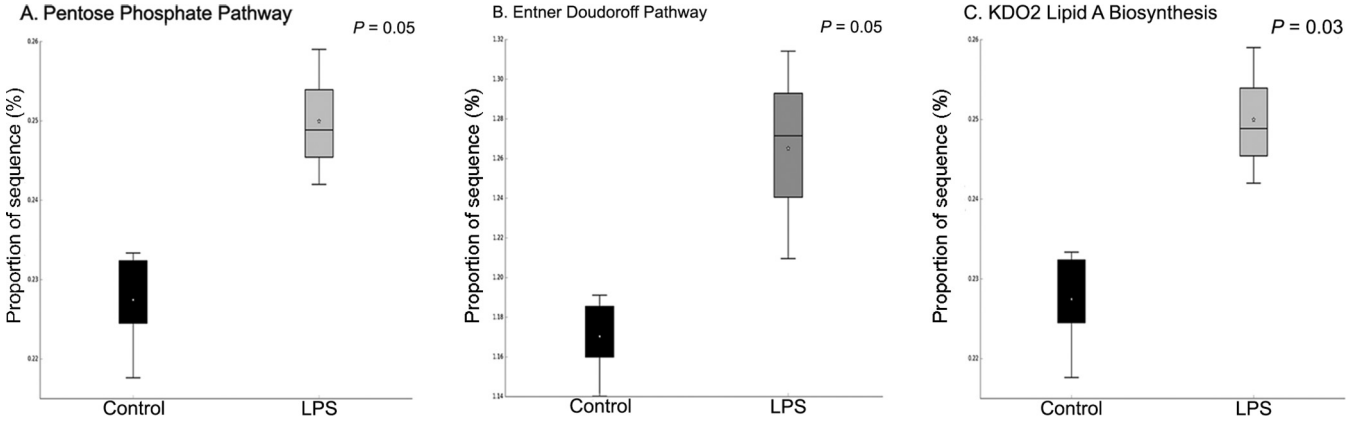

FIG 3 Effects of lipopolysaccharide dosing on the proportion of sequence ssociated with the pentose phosphate pathway (A; KEGG), Entner-Doudoroff pathway (B; SEED), and KDO_2-lipid A biosynthesis (C; SEED) level 3.

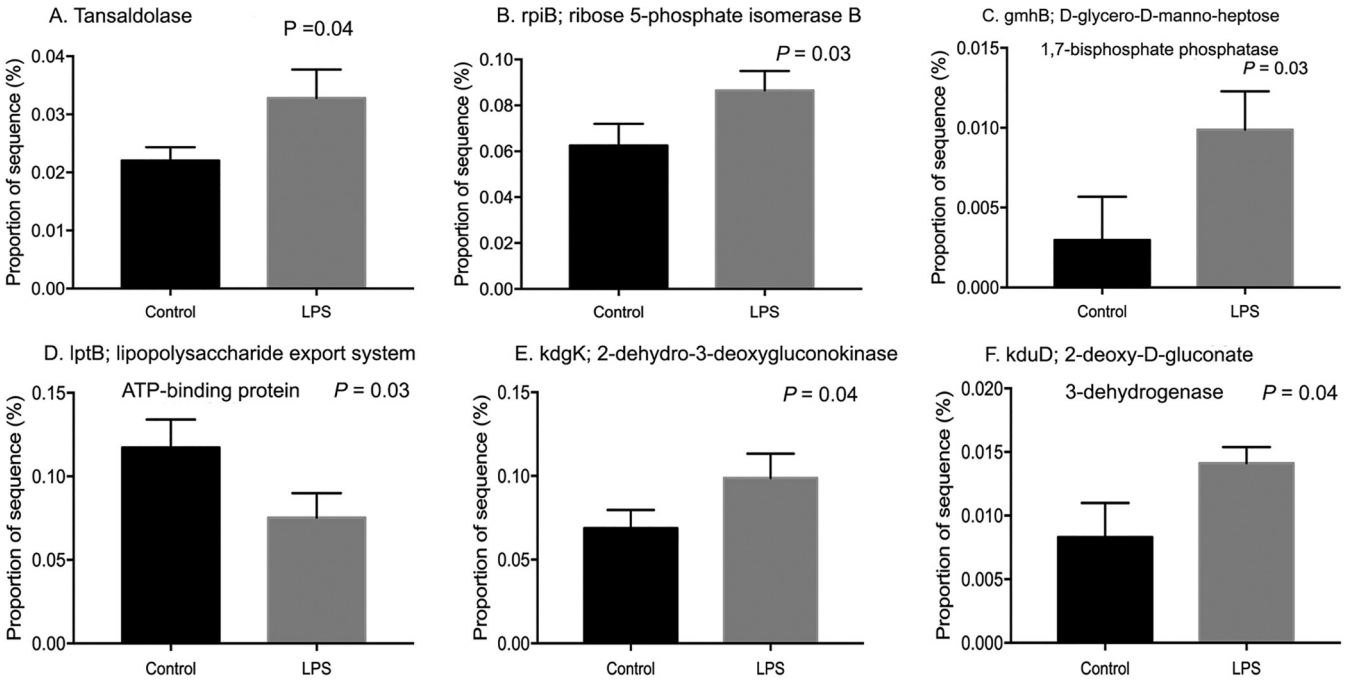

FIG 4 Effects of lipopolysaccharide dosing on the proportion of sequence of functional genes associated with the transaldolase gene (A), *rpiB* (B), *gmhB* (C), *lptB* (D), *kdgK* (E), and *kduD* (F) based on SEED (B) and KEGG (A and C to F) data sets.

another soluble carbon source (e.g., starch or glucose) present. The fermentation end products of all the tested bacteria were not affected by LPS dosing. Therefore, the hypothesis that LPS is used as the carbon source for lactate-producing bacteria and thus stimulates their growth was rejected; otherwise, due to the additional carbon source (LPS) in the medium, the fermentation end products may be increased by LPS dosing.

We thought that the presence of LPS may work as a factor to stimulate the growth of lactate-producing bacteria. It has been reported that LPS is essential for all tested ruminal strains of *Anaeroplasma abactoclasticum* (a starch-producing ruminal bacterium) in pure culture and 0.25 mg/liter of LPS stimulated growth of strain 6-1 (21). Unfortunately, the mechanism of how LPS stimulated *Anaeroplasma abactoclasticum*

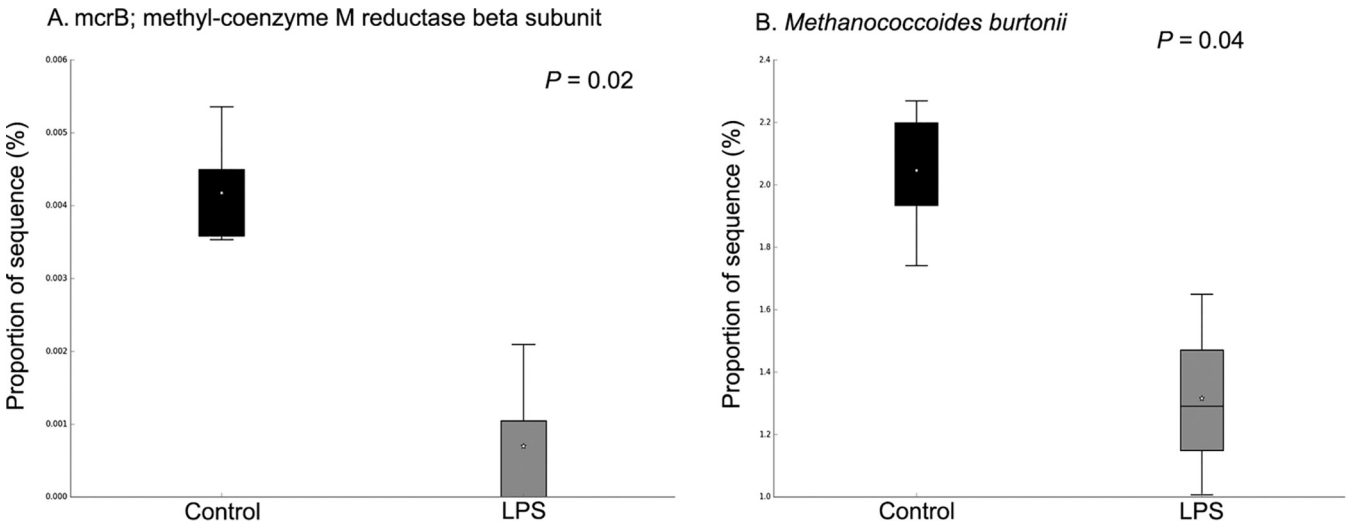

FIG 5 Effects of lipopolysaccharide dosing on the proportions of sequences associated with *mcrB* (A) and *Methanococcoides burtonii* (B).

6-1 was not elucidated from the study. LPS is composed of a lipid A moiety, inner and outer core oligosaccharides, and the O antigens, where lipid A consists of fatty acyl chains and the rest are polysaccharides (20). We questioned which part of LPS (fatty acyl chain or polysaccharide) could potentially act to stimulate the growth of lactate-producing bacteria. To evaluate this, the same concentrations of delipidated LPS (lipid A delipidated) and intact (regular) LPS were tested for their effects on the growth of *S. bovis* JB1. We found that when LPS was mainly present as polysaccharide (lipid A delipidated), the maximum specific growth rate of *S. bovis* JB1 was increased by only 7.5% and the lag phase was not affected. However, the maximum specific growth rate was increased by 15% and the lag phase was decreased by 42 min by the regular/intact LPS (lipid A present). Therefore, the stimulating effects of LPS on the growth of *S. bovis* JB1 could mainly contribute to the presence of lipid A of LPS. Future studies could perhaps focus on evaluating the effects of pure lipid A of LPS on the growth of lactate-producing bacteria to validate this.

Meanwhile, lipid A, covalently linked to the inner core, carries the endotoxic activity of LPS (1). We thought that LPS may be toxic to other non-lactate-producing ruminal bacteria, increasing the competition of lactate-producing bacteria and thus stimulating their growth. *Megasphaera elsdenii* T81 was chosen because it is the main lactate-utilizing ruminal bacterium and is dominantly present in ruminal acidosis. *Megasphaera elsdenii* T81 treated with the same concentrations of detoxified (lipid A delipidated) and regular LPS was used to test this hypothesis. Removal of the fatty acid portions of lipid A results in a detoxified LPS with an endotoxin level about 10,000 times lower than that of the regular LPS based on the Sigma-Aldrich protocol. However, there were no difference on the maximum specific growth rate and lag phase of *M. elsdenii* T81 among control, detoxified LPS, and regular LPS dosing. Meanwhile, *S. bovis* JB1 had a lower maximum specific growth rate with detoxified LPS than with regular LPS. Therefore, LPS may have no toxic effects on ruminal bacteria. In turn, the endotoxin carrier—lipid A—may act as a factor to stimulate the growth of lactate-producing bacteria.

Shotgun sequencing was performed to evaluate the effects of LPS dosing on the metabolic capabilities of ruminal bacteria. It was observed that dosing of LPS increased the sequence abundances of microbial functional genes associated with LPS biosynthesis pathway. KDO_2-lipid A and ADP-D-glycero-β-D-manno-heptose are two necessary precursors for LPS biosynthesis (22). The sequence abundances of functional genes associated with KDO_2-lipid A biosynthesis were increased by LPS dosing; the sequence abundance of *gmhB* involved in ADP-D-glycero-β-D-manno-heptose biosynthesis was also increased by LPS dosing. Meanwhile, the sequence abundances of microbial functional genes associated with *rpiB* and the transaldolase gene, which are involved in D-ribulose-5-phosphate (D-ribulose-5P) and D-sedoheptulose-7-phosphate synthesis, respectively, were increased by LPS dosing. D-Ribulose-5-phosphate and D-sedoheptulose-7-phosphate could initiate LPS biosynthesis (23), which could be converted into KDO_2-lipid A biosynthesis (24) and ADP-L-glycero-β-D-manno-heptose (25) to synthesis, respectively (23, 24, 26). Sequence abundances of microbial functional genes associated with *kdgK* and *kduD*, which are involved in the synthesis of 2-dehydro-3-deoxy-6-phospho-D-gluconate (the precursor of D-ribulose-5P) based on the PPP pathway and LPS biosynthesis pathway, were increased by LPS dosing. Therefore, dosing of LPS mainly increased the microbial functional genes associated with the LPS biosynthesis pathway, and microbial functional genes associated with the PPP and EDP were increased by LPS dosing to increase the precursors for LPS biosynthesis.

However, the sequence abundance associated with LPS export system ATP-binding protein (*lptB*) was decreased by LPS dosing. The energy responsible for driving LPS transport is derived from ATP hydrolysis catalyzed by *lptB* (20, 27, 28). This energy must be coupled to move across the periplasm and outer membrane (OM) in Gram-negative bacteria, as there is no ATP in these compartments (20). *In vivo* studies demonstrated that all Lpt proteins are required for the transport of LPS; depletion of any of the Lpt proteins leads to the accumulation of LPS in the periplasmic leaflet of the inner membrane (IM) (29, 30). Therefore, sequence abundance of *lptB* was decreased by LPS

dosing, which may lead to the accumulation of LPS in the periplasmic leaflet of IM of bacteria. LPS accumulation in the IM of bacteria may increase the osmotic pressure and then may increase LPS release from bacteria into the rumen.

The increase of the sequence abundance associated with LPS biosynthesis may be due to the increase in the sequence abundance of Gram-negative bacteria by LPS dosing. With the increase of Gram-negative bacteria by LPS dosing, the demands of LPS biosynthesis for outer membrane assembly were increased. Therefore, the sequence abundances of functional genes associated with the LPS biosynthesis pathway were increased. Our previous study also reported that LPS dosing mainly stimulated the Gram-negative bacteria related to starch digestion (the genera *Succinimonas*, *Anaeroplasma*, *Succinivibrio*, *Succiniclasticum*, and *Ruminobacter*) (14). In turn, LPS dosing-increased growth of Gram-negative bacteria may be due to stimulation of functional genes involved in the LPS biosynthesis pathway. However, in order to validate how exactly LPS affects the LPS biosynthesis pathway, collecting RNA samples from the single strains of Gram-negative bacteria and analyzing the genes involved in LPS biosynthesis by quantitative PCR (qPCR) or transcriptome sequencing (RNA-seq) would be suggested.

Sequence abundances of functional genes associated with the *methyl*-coenzyme M reductase beta subunit (*mcrB*) involved in methane metabolism were decreased by LPS dosing. The enzyme encoded by the *mcrB* gene catalyzes the reductive cleavage of *methyl*-coenzyme M [CoM-S-CH3; 2-(methylthio)ethanesulfonate] using coenzyme B (CoB; 7-mercaptoheptanoylthreonine phosphate) as reductant and produce methane and the mixed heterodisulfide of CoB and CoM (CoM-S-S-CoB) (31). This is the final step in methanogenesis. At the same time, the sequence abundance of *Methanococcoides burtonii* was decreased by LPS dosing as well. *Methanococcoides burtonii* is known as a methanogen; methanogens produce methane by decomposition of organic carbons under anaerobic conditions (32). Therefore, dosing of LPS might decrease methane production by directly affecting methanogen and the functional genes involved in methane formation. In the future, studies could focus on increased ruminal LPS concentration as a function of the growth or lysis of Gram-negative bacteria and thus help to understand and possibly develop practical strategies to control ruminal methane emissions.

Conclusion. Dosing of LPS mainly stimulated the growth of lactate-producing bacteria (*S. bovis* JB1, *Se. ruminantium* HD4, *Su. dextrinosolvens* 24, and *L. ruminis* RF1), regardless of Gram reaction, and not fiber-utilizing bacteria (*R. albus* 7, *R. flavefaciens* FD-1, and *F. succinogenes* S85) or a lactate-utilizing bacterium (*M. elsdenii* T81). Lipopolysaccharide could not replace glucose as a carbon source for lactate-producing bacteria (*S. bovis* JB1), and stimulating effects of LPS on the growth of lactate-producing bacteria were observed only when there was another soluble carbon source (e.g., starch or glucose) present. Dosing of LPS has no toxic effects on tested bacteria, and the presence of lipid A or intact LPS was the main contributor to the stimulating effects of LPS on the growth of lactate-producing bacteria. The sequence abundances of functional genes associated with the PPP, EDP, and LPS biosynthesis pathway were increased by LPS dosing, while the sequence abundance of functional genes involved in methanogenesis and sequence abundance of *Methanococcoides burtonii* were decreased by LPS dosing. In summary, LPS stimulated the growth of ruminal bacteria that use starch and produce lactate. Our results indicate that LPS not only is increased during acidosis but also may contribute to ruminal acidosis development by increasing the growth of lactic acid-producing bacteria; dosing of LPS may stimulate the LPS biosynthesis of ruminal bacteria.

MATERIALS AND METHODS

Organisms. Fiber-utilizing bacteria (*Ruminococcus albus* 7 (Gram negative), *Ruminococcus flavefaciens* FD-1 (Gram negative), *Fibrobacter succinogenes* S85 (Gram negative), lactate-producing bacteria (*Streptococcus bovis* JB1 [Gram positive], *Succinivibrio dextrinosolvens* 24 [Gram negative], *Lactobacillus ruminis* RF1 [Gram positive], and *Selenomonas ruminantium* HD4 [Gram negative]), and a lactate-utilizing bacterium (*Megasphaera elsdenii* T81 [Gram negative]) were chosen for the current study. The Gram

characteristic was based on the Gram reaction. *Ruminococcus albus* 7, *R. flavefaciens* FD-1, *F. succinogenes* S85, and *M. elsdenii* T81 were obtained from P. Weimer (USDA-ARS, Madison, WI). *Streptococcus bovis* JB1 and *Su. dextrinosolvens* 24 were obtained from the ATCC. *Lactobacillus. ruminis* RF1 was obtained from the DSMZ. *Selenomonas ruminantium* HD4 was obtained from Michael Flythe (USDA-ARS, Lexington, KY). The bacteria were preserved in anaerobic dilution solution with 50% glycerol in Balch tubes with butyl rubber stoppers and aluminum crimp seals and stored at −20°C.

Maintenance and growth of strains. For each experimental run, each strain was revived from the Balch tube that was stored at −20°C and transferred twice in the same fresh media as the ones used before experiments started. The stored bacteria can be freeze-thawed multiple times without effect on viability of bacteria (33). During the experiment, 0.1 ml of homogeneous stock culture was transferred from the previous stock culture by a 1-ml syringe with a 22-gauge needle into fresh medium vials that were sealed with a flanged butyl rubber stopper and aluminum crimp seal. Anaerobic water and regular and detoxified LPS were also transferred into the Balch tubes by a 1-ml syringe with a 22-gauge needle after transfer of bacteria. The glucose stock was added to Balch tubes of defined medium right before transfer of bacteria. All of the procedures were done on a disinfected bench with 75% ethanol and under anaerobic conditions.

Unless otherwise stated, strains were grown anaerobically under O_2-free CO_2 in Balch tubes with butyl rubber stoppers and aluminum crimp seals and incubated at 39°C without shaking. The optical density (OD) before (OD_b) and after (OD_0) bacterium inclusion were measured at 600 nm on spectrophotometer (Genesys 20; Thermo Scientific, Waltham, MA) for each experimental run and for each strain. Measurements of OD at 600 nm (OD_t) were collected every hour except in the case of *S. bovis* JB1, for which measurement s were collected every 30 min until bacterial growth reached a plateau. The real OD of strains was calculated as $OD_t - OD_b$. Once bacterial growth reached a plateau, the culture vials were decapped and stock culture medium was collected and stored in −20°C for later determination of the fermentation end products (NH_3-N, VFA, and lactate).

Media. Phosphate carbonate minus VFA (PC–VFA) medium contained 4 g of glucose, 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 49 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4 g of Na_2CO_3, 1 g of Trypticase peptone (product 212750; BD), and 0.5 g of yeast extract (product 212750; BD) per liter (34, 35). Resazurin was added as a redox indicator. Strains grown on PC–VFA included *S. bovis* JB1, *M. elsdenii* T81, and *Se. ruminantium* HD4.

In order to stimulate growth, short-chain fatty acids were added to the above-described medium, giving PC+VFA. Short-chain fatty acids included 1.7 ml of acetic acid, 0.6 ml of propionic acid, 0.4 ml of *n*-butyric acid, 0.1 ml of *n*-valeric acid, 0.1 ml of isovaleric acid, 0.1 ml of isobutyric acid, and 0.1 ml of 2-methylbutyric acid per liter (15, 34, 35). Strains grown on PC+VFA included *L. ruminis* RF1 and *Su. dextrinosolvens* 24. In order to grow fiber-utilizing bacteria (*F. succinogenes* S85, *R. albus* 7, and *R. flavefaciens* FD1), 4 g of glucose in PC+VFA medium was replaced with 3 g of cellobiose per liter of medium (34).

To determine if LPS would replace glucose as carbon source for the growth of *S. bovis* JB1, a defined medium was modified from PC-VFA medium (36, 37) in which, per liter of the medium, 4 g of glucose, 1 g of Trypticase peptone, and 0.5 g of yeast extract were removed and 5 ml of Pfennings heavy metal solution, 1 ml of hemin solution, and 10 ml of Russell vitamin solution were added to the medium. Pfennings heavy metal solution included 0.5 g of EDTA disodium salt, 0.2 g of $FeSO_4 \cdot 7H_2O$, 0.2 g of $MnCl_2 \cdot 4H_2O$, 0.01 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of H_3BO_4, 0.02 g of $CoCl_2 \cdot 6H_2O$, 0.001 g of $CuCl_2 \cdot 2H_2O$, 0.002 g of $NiCl_2 \cdot 6H_2O$, and 0.003 g of $NaMoO_4 \cdot 2H_2O$ per liter (38). Hemin solution included 2.8 g of KOH, 250 ml of 95% ethanol, and 1 g of hemin per liter (39). Russell vitamin solution included 0.1 g of pyridoxamine dihydrochloride, 0.1 g of pyridoxal hydrochloride, 0.1 g of pyridoxine, 0.2 g of riboflavin, 0.2 g of thiamine HCl, 0.2 g of nicotinamide, 0.2 g of calcium pantothenate, 0.1 g of lipoic acid, 0.01 g of 4-aminobenzoic acid, 0.005 g of folic acid, 0.005 g of biotin, and 0.005 U of coenzyme B_{12} in 1 liter of 0.1 M KH_2PO_4 (36; M. Flythe, personal communication).

Anaerobic water, LPS, and glucose stock preparation. (i) Anaerobic water. Nonpyrogenic water (catalog no. W50-500; Lonza Group Ltd., Basel, Switzerland), was boiled for 5 to 7 min and dispensed into culture tubes while flushing with N_2, and cultured tubes were capped with butyl rubber stoppers and aluminum crimp seals for autoclaving.

(ii) LPS stock. The concentrations of regular LPS (*Escherichia coli* O111:B4, L2630; Sigma-Aldrich Co., St. Louis, MO) and delipidated/detoxified LPS (lipid A delipidated, *Escherichia coli* O111:B4, L3023; Sigma-Aldrich Co.) were 200,000 EU, the same as in our previous study (14). The LPS was prepared under anaerobic conditions, where 100 mg of LPS was added to 250 ml of anaerobic nonpyrogenic water while flushing with N_2, to generate 0.4 mg/ml of LPS. The LPS stock was then filtered through a 0.22-μm polyethersulfone (PES) membrane syringe filter (Celltreat, Pepperell, MA) into Balch tubes that were previously flushed with N_2 and autoclaved. A 0.5-ml volume of 0.4-mg/ml regular or detoxified LPS stock was equal to 200,000 EU of LPS (1 ng/ml = 10 endotoxin units based on the Sigma-Aldrich protocol, the volume of culture media in Balch tubes of 10 ml).

(iii) Glucose stock. Glucose stock was prepared under anaerobic conditions, where 4.2 g of glucose was added to 70 ml of anaerobic nonpyrogenic water while flushing with N_2, to generate 0.06 g/ml of glucose. The glucose stock was then filtered through a 0.22-μm PES membrane syringe filter into 160-ml culture tubes with flanged butyl stoppers and aluminum crimp seals that were previously flushed with N_2 and autoclaved. The glucose stock concentration was the same as in the PC−VFA and PC+VFA media.

Experimental design and measurement of bacterial growth. (i) Experiment 1. The goal of experiment 1 was to evaluate effects of regular LPS (200,000 EU) on fiber-utilizing bacteria (*R. albus* 7, *R. flavefaciens* FD-1, and *F. succinogenes* S85), lactate-producing bacteria (*S. bovis* JB1, *Su. dextrinosolvens* 24,

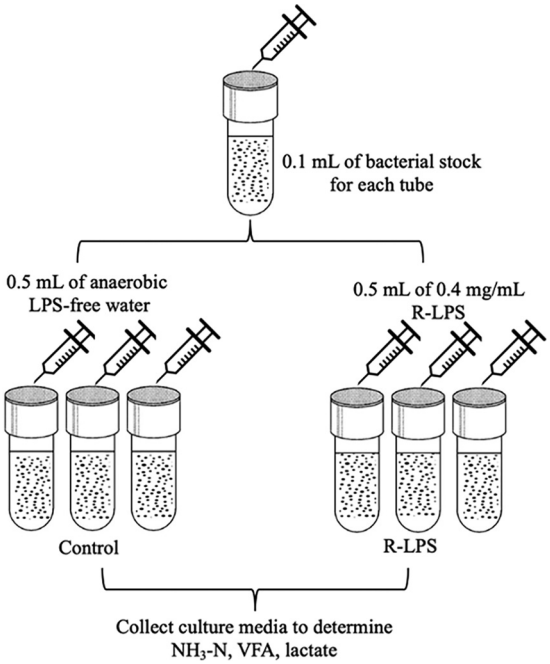

FIG 6 Experimental procedure flowchart, experiment 1. Control, control group (LPS-free anaerobic water); R-LPS, regular LPS (200,000 EU).

L. ruminis RF1, and *Se. ruminantium* HD4), and a lactate-utilizing bacterium (*M. elsdenii* T81). Treatments were (i) a control (0.5 ml of anaerobic water) and (ii) regular LPS (0.5 ml of 0.4-mg/ml regular LPS). There were at least three experimental runs per strain, and each treatment had three replications. The experimental procedure flowchart is presented in Fig. 6.

(ii) Experiment 2. The goal of experiment 2 was to evaluate if LPS (200, 000 EU) could replace glucose as a carbon source for *S. bovis* JB1 growth with the defined medium. Treatments were (i) a control (1 ml of anaerobic water), (ii) LPS (0.5 ml of anaerobic water plus 0.5 ml of 0.4-mg/ml regular LPS), (iii) GLU (0.5 ml of anaerobic water plus 0.5 ml of 0.06 mg/ml glucose), and (iv) GLU plus LPS (0.5 ml of 0.4-mg/ml regular LPS plus 0.5 ml of 0.06-g/ml glucose). Once growth of *S. bovis* JB1 in GLU and GLU plus LPS reached a plateau, *S. bovis* JB1 did not grow in control and LPS media; 0.5 ml of 0.06-g/ml glucose was then added into control and LPS tubes to measure the growth of *S. bovis* JB1. There were five experimental runs, and each treatment had three replications. The experimental procedure flowchart is presented in Fig. 7.

(iii) Experiment 3. The goal of experiment 3 was to evaluate the toxicity of LPS (200,000 EU) on *S. bovis* JB1 in a defined medium with glucose added as a carbon source and *M. elsdenii* T81 in PC−VFA medium. Treatments were (i) a control (0.5 ml of anaerobic water), (ii) R-LPS (0.5 ml of 0.4-mg/ml regular LPS), and (iii) D-LPS (0.5 ml of 0.4-mg/ml detoxified LPS). There were five experimental runs per strain, and each treatment had three replications. The experimental procedure flowchart is presented in Fig. 8.

Ammonia, VFA, and lactate analyses. (i) Sample preparation. Ten milliliters of stock culture medium used for NH_3 and VFA concentration analyses was preserved with 0.1 ml of 50% H_2SO_4. After that, samples were centrifuged at 10,000 × *g* for 15 min at 4°C. One milliliter of supernatant was filtered through a 0.22-μm syringe filter (Tisch Scientific, North Bend, OH) into 2-ml vials used for VFA analysis, and the rest of acidic samples were preserved for NH_3-N analysis.

(ii) Ammonia nitrogen concentration analysis. Ammonia nitrogen concentration analysis was adapted from Broderick and Kang (40). Briefly, 2 μl of samples, 100 μl of phenol, and 80 μl of hypochlorite were added to each well of a flat-bottom 96-well plate, mixed well, and then incubated at 95°C for 5 min. After cooling, the plate was read at 620 nm on a microplate spectrophotometer (Spectra Max 340 PC; Molecular Devices Corporation, Sunnyvale, CA).

(iii) Volatile fatty acid analysis. Concentrations of VFA in ruminal fluid samples were determined in a liquid-liquid solvent extraction using ethyl acetate (41). Ruminal fluid supernatant was mixed with a *meta*-phosphoric acid–crotonic acid (internal standard) solution at a 5:1 ratio, and samples were frozen overnight, thawed, and centrifuged for 15 min at 10,000 × *g*. Supernatant was transferred into glass tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. After the tubes were shaken vigorously and the fractions were allowed to separate, the ethyl acetate fraction (top layer) was transferred to vials. Samples were analyzed by gas chromatography (Agilent 7820A gas chromatograph; Agilent Technologies, Palo Alto, CA) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP, 25 m by 0.53 mm, Varian CP7767; Varian Analytical Instruments, Walnut Creek, CA). Column temperature was maintained at 110°C, and injector and detector temperatures were 200 and 220°C, respectively.

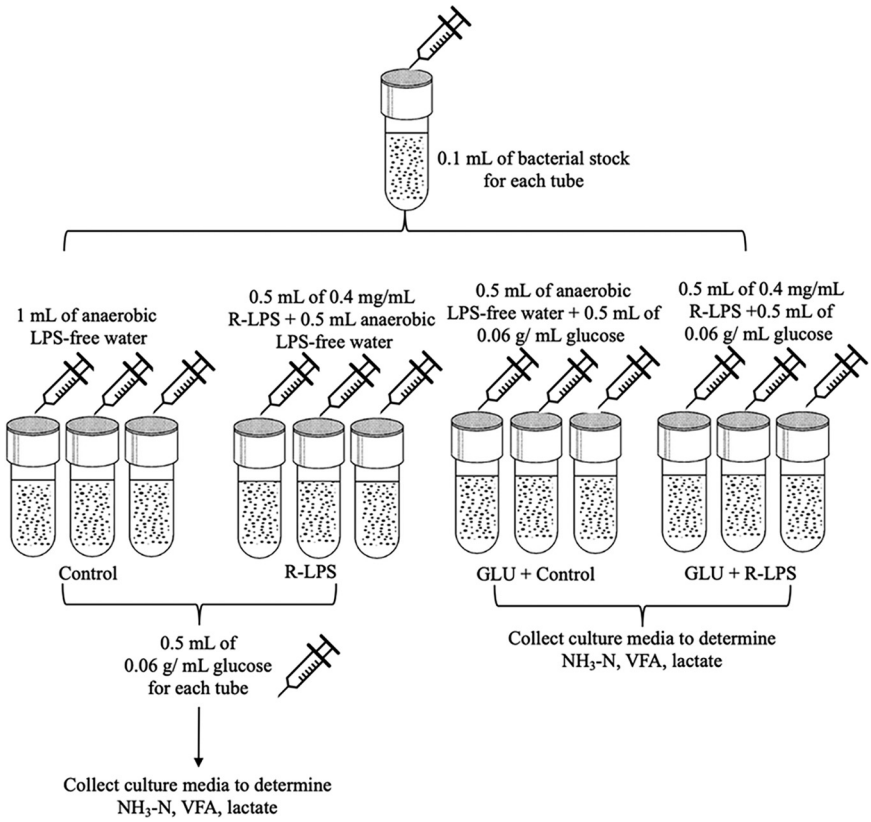

FIG 7 Experimental procedure flowchart, experiment 2. Control, control group (LPS-free anaerobic water); LPS, regular LPS (200,000 EU) as a carbon source; GLU, glucose as a carbon source; GLU + LPS, glucose plus LPS (200,000 EU).

(iv) Lactate analysis. The stock culture medium used for lactate analysis was first boiled at 100°C for 10 min and then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were then used for lactate analysis. The UV method was used to determine D- and L-lactic acid concentrations by using a D-lactic acid/L-lactic acid kit from R-Biopharm AG (Darmstadt, Germany). Concentrations of D- and L-lactic acid were analyzed on a flat-bottom 96-well plate and read at 340 nm on a microplate spectrophotometer. The analysis procedure was described in detail in reference 14.

Shotgun sequencing and metagenomic data processing. (i) Library preparation and sequencing. In order to evaluate the effects of LPS on metabolic capabilities of ruminal bacteria, we dosed 200,000 EU of R-LPS in a dual-flow continuous culture system that was designed to mimic the ruminal microbial fermentation. There were four fermentors randomly assigned within Latin square to receive either control or regular LPS (200,000 EU) over 3 periods with 7 days of diet adaptation followed by 4 days of bacterial sampling per period. Bacterial samples were collected at 2, 5, 7, and 9 h after morning feeding per sampling day. Then the same amount of bacterial sample from the same treatment and period were pooled for both control and LPS treatments. There were three replications per treatment, and a total of 6 bacterial samples were processed for the DNA isolation and shotgun sequencing. The experimental diets, sample collection, and DNA extraction processing were described in detail in reference 14.

A Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) was used to measure the DNA concentration of each sample, and each sample was diluted to around 0.2 ng/μl for library preparation. Libraries were prepared for sequencing by using a Nextera XT DNA library prep kit (FC-131-1001; Illumina Inc., San Diego, CA). The libraries were constructed according to the Nextera XT DNA library prep kit reference guide (document number 15031942 v03; February 2018). The generated library plus 1% PhiX spike in control and were sequenced on Illumina Miseq using MiSeq reagent kit V2 (2 × 250 cycles; Illumina) according to the manufacturer's protocol.

(ii) Shotgun metagenomic data processing. The paired-end sequences obtained were merged by Sickle (42), and low-quality bases (quality score lower than 20) and sequences below 50 nucleotides were removed. Metagenome assembly was done by metaSPAdes with *k*-mers of 33, 55, and 77 (43). The assembly sequences were annotated with the Metagenomics Rapid Annotation (MG-RAST) pipeline, version 4.0.3 (44). Functional profiles were generated using the normalized abundance of sequence matches to the SEED (45) and KEGG (46) databases, respectively. A table of the frequency of hits to each individual function for each metagenome was generated and normalized. To identify hits, BLASTX was used (47) with a minimum alignment length of 50 bp, minimal identity of 60%, and an E value cutoff of $<1 \times 10^{-5}$. The matrices generated were exported and used for statistical analyses.

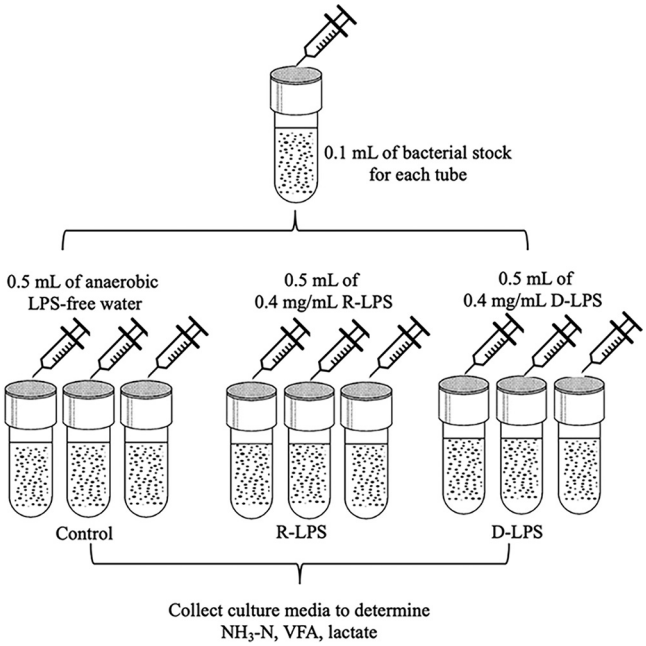

FIG 8 Experimental procedure flowchart, experiment 3. Control, control group (LPS-free anaerobic water); R-LPS, regular LPS (200,000 EU); D-LPS, detoxified LPS (200,000 EU).

Statistical analysis. Logistic function was used to predict the growth rate (μ) and lag phase (lag), as the R^2 values were all greater than 99.5% and MSE were lower than 0.001 for each experimental run of tested strains. According to reference 48, the logistic function used in the study was

$$Y = y_0 + \frac{C}{(1 + exp^{[4\times\mu\max\times(\mathrm{lag}-t)/C+2]})}$$

where Y is real OD_t, y_0 is initial OD_0, μmax is maximum specific growth rate, and C is increase of OD from OD_0 to OD_t.

The logistic model was fit to the data by nonlinear regression using NLIN procedure of SAS software (version 9.4; SAS Institute Inc., Cary, NC). Several possible starting values were specified for each parameter, so that the NLIN procedure evaluated the model at each combination of initial values on the grid, using for the first iteration of the fitting process the combination yielding the smallest residual sum of squares. The initial values supplied were different per run per strain, and the selection of the starting values was based on visual inspection of the growth curve. The uniqueness of the final solution achieved in each case was checked by changing the initial parameter estimates within a reasonable range of each data set.

The predicted maximum specific growth rate and lag phase were used as input for SAS analysis. The effects of treatment on maximum specific growth rate and lag phase, and concentrations of ammonia, VFA, and lactate were subjected to least-square analysis of variance (ANOVA) using the MIXED procedure of SAS. All data were checked for the linear model assumption. The statistical model used was

$$y_i = \mu + T_i + E_j + \varepsilon_{ij}$$

where y is a dependent variable, μ is overall mean, T_i is fixed effect of treatments,E_j is experimental run, and ε_{ij} is the random error. Experimental run was considered the random effect. Different covariance structures were tested, and the one with the lowest Akaike information criterion values were chosen.

The statistical differences between the sequencing data were determined using the Statistical Analysis of Metagenomic Profiles v2.1.3 (STAMP) software (49). For functional profiling, a table with hit frequency for each metagenome was generated from MG-RAST and used as input. P values were calculated using two-sided Welch's t test (50). Corrected P values below 0.05 were considered significant. Data will be made fully available and without restriction.

Data availability. All sequences from this project have been deposited at the National Center for Biotechnology Information Sequence Read Archive under accession number SRP216606.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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We declare no conflicts of interest.

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