Use of a novel oleaginous microorganism as a potential source of lipids for weanling pigs[1,2]

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ABSTRACT: Weanling pigs are at risk of succumbing to illness due to an immature immune system and insufficient supply of available energy at the time of weaning. This study was aimed at determining whether oleaginous bacteria could serve as a source of lipids to weanling pigs. Weanling pigs were provided a daily dose of 1×10^9 colony fomring unit (CFU) = kg^{-1} of the novel oleaginous *Enterobacter cloacae* strain JD6301 or JD8715 (which is a variant form of JD6301 capable of producing extracellular triglycerides) via oral gavage for 5 d. Serum was collected every 6 h and intestinal samples were collected at 6 d. Providing pigs with JD6301 or JD8715 significantly increased serum concentrations of triglycerides and non-esterified fatty acids (NEFA) within 72 h. Additionally, the JD6301 and JD8715 strains were able to survive within the gastrointestinal tract throughout the duration of the study. These results suggest that providing *Enterobacter cloacae* can increase the serum lipids in the pigs, thus potentially providing an additional source of energy to animals during times of stress. This could potentially help improve the metabolic response of animals during times of stress.

Key words: *Enterobacter cloacae,* lipids, oleaginous bacteria, triglycerides

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INTRODUCTION

Animal health is one of the primary concerns among livestock producers, with an estimated $329 million in annual losses associated with illnesses among pre-weaning swine (Lay et al., 2002). The primary causes for these losses are due to infections and increased energy demands in livestock with low energy reserves (Lay et al., 2002). Weanling pigs are particularly more susceptible to illness due to reduced energy metabolism, limited energy reserves (i.e adipose tissue), and decreased feed intake, which results in an inefficient immune response.

The normal flora of the gastrointestinal tract (GIT) influences the gut immune system and also prevents colonization of harmful pathogens (Hooper and Gordon 2001). However, an imbalance of this delicate system can lead to dysbiosis (Cerf-Bensussan and Gaboriau-Routhiau 2010). Commensal microorganisms can stimulate the immune system, enabling a more efficient response to foreign entities (Mazmanian et al., 2005). The stimulation of the immune system requires interactions between Toll like receptors and lipids for both activation and energy supplies (Aderem and Ulevitch 2000). These lipids can originate from

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the diet or from reserves within adipose tissue, and are essential sources of energy for the immune system (Pond and Mattacks 1995). Infusion with lipids has been shown to improve the response of animals to endotoxins (Feingold et al., 1995, Read et al., 1995). A limitation exists in the application of this mechanism during times of depressed appetite, such as during periods of stress, illness, or in animals with limited energy reserves as observed in very young animals.

A possible solution to increase the availability of lipids is to supplement the diet or water source with microorganisms capable of producing large amounts of lipids. Oleaginous microorganisms are able to produce at least 20% of the total cell weight as lipids. *Enterobacter cloacae* strain JD6301 was described as a novel oleaginous bacterium capable of producing up to 50% of the cell weight in lipids (Donaldson et al., 2014, Wilson et al., 2014). A modified form of this strain, termed JD8715, produces extracellular lipids (Donaldson et al., 2014). Therefore, the objective of this study was to determine if *Enterobacter cloacae* strain JD6301 and JD8715 increase lipid availability to weanling pigs.

MATERIALS AND METHODS

All animal procedures were reviewed and approved by the USDA-ARS-Livestock Issues Research Unit's Animal Care and Use Committee (IACUC #2012–02-JAC12).

Bacterial Strains and Conditions

Enterobacter cloacae strains JD6301 and JD8715 were previously characterized (Donaldson et al., 2014, Wilson et al., 2014). Both strains were routinely cultured onto nutrient agar (NA) supplemented with 50 μg mL^{-1} novobiocin at 37°C. Isolated colonies were cultured in 25 mL of Mineral Salts Media with 3% glucose and 0.1 g l^{-1} NH_4SO_4 for 24 h at 37°C as previously described (Alvarez et al., 1996). Cells were then pelleted through centrifugation at 10,000 × *g* for 5 min and resuspended in 20 mL of phosphate buffered saline (PBS; ~2 × 10^9 CFU mL^{-1}).

For growth analysis of JD6301 and JD8715, cells were cultured overnight in nutrient broth (NB) at 37°C, then diluted 1:100 in 0.2 mL of NB supplemented with 0, 5, or 10% porcine bile salts (Oxgall, Sigma #8008) or NB at an adjusted pH of 6.0, 5.0, or 4.0. The OD_{600} measurements were collected hourly with a BioTek Powerwave HT microplate spectrophotometer at 37°C (Winooski, VT).

Animals and Design

Forty male weanling *Sus scrofa domesticus* (21 d of age, average 6 kg) were purchased from a commercial farm and transferred to an environmentally controlled swine facility (Liberty Farm, Lubbock, TX). Pigs were individually housed and allowed to acclimate to the facility for 10 d prior to being non-surgically fitted with jugular catheters using methods previously described (Carroll et al., 1999). Fecal samples were also collected prior to beginning the supplementation to confirm sensitivity to 50 μg mL^{-1} novobiocin. Pigs were stratified by body weight and randomly assigned to 1 of 3 treatment groups (n = 12 per group): 1) PBS; 2) JD6301 in PBS (~1x10^9 CFU kg^{-1}); or 3) JD8715 in PBS (~1×10^9 CFU kg^{-1}). On d 0, pigs in each treatment group received their respective treatments via an oral gavage (~2 mL dose). Treatments were provided by oral gavage every 24 h for 5 d. Serum samples were collected every 6 h for 4 d. On d 6, pigs were humanely sacrificed and contents from the cecum, jejunum, ileum, and rectum were collected into sterile conical tubes. Intestinal samples were serially diluted into PBS and plated onto NA supplemented with 50 μg mL^{-1} novobiocin. Plates were incubated at 37°C under anaerobic conditions. Fecal samples were also collected daily. Briefly, approximately 1 g of feces was added to 1 mL PBS, vortexed to resuspend, and serially diluted into PBS. Dilutions were plated onto NA supplemented with 50 μg mL^{-1} novobiocin and incubated at 37°C.

Serum Analysis

Serum was analyzed in duplicate for all assays. Serum concentrations of triglycerides (TAG) were measured using the Triglyceride Colorimetric Assay kit (Cayman Chemical, Ann Arbor, MI) per the manufacturer's protocol. Briefly, 10 μL of serum was added to 150 μL of enzyme buffer and incubated for 15 min at room temperature. Colorimetric readings were recorded using a BioTek PowerWave plate reader (Winooski, VT) at 550 nm. Concentrations were determined by comparing the unknown serum samples to a standard curve of known triglyceride concentrations (Cayman Chemical).

Free glycerol was measured in serum using the Glycerol Colorimetric Assay kit (Cayman Chemical) per manufacturer's instructions. Briefly, 10 μL of serum was added to 150 μL of the enzyme buffer and incubated for 15 min at room temperature. Colorimetric readings were recorded using a BioTek PowerWave plate reader at 550 nm. Concentrations were determined by comparing the unknown serum samples to a standard curve of known glycerol concentrations.

Serum concentrations of glucose were determined using the Glucose Colorimetric Assay kit (Cayman Chemical) per manufacturer's instructions. Briefly, 15 μL of serum was added to 85 μL of diluted assay buffer and 100 μL of enzyme mixture. Reactions were incu-

bated for 10 min at 37°C. Colorimetric readings were recorded using a BioTek PowerWave plate reader at 505 nm. Concentrations were determined by comparing the unknown serum samples to a standard curve of known glucose concentrations.

Non-esterified fatty acids (NEFA) were quantitated by the NEFA-HR commercial kit (Wako Diagnostics, Richmond, VA). Briefly, 10 µL of serum was added to 300 µL of reagent buffer 1 and 150 µL of buffer 2. Samples were incubated for 5 min at 37°C prior to being measured with a BioTek PowerWave plate reader at 550 nm. Concentrations were determined against a standard curve of known NEFA concentrations.

Lipid Composition Analysis

Lipids were extracted from serum samples using the method of Folch-Lees (Folch et al., 1957). The extracts were filtered and lipids recovered in the chloroform phase. Individual lipid classes were separated by thin layer chromatography using Silica Gel 60 A plates developed in petroleum ether, ethyl ether, acetic acid (80:20:1) and visualized by rhodamine 6G. Phospholipids, diglycerides, triglycerides and cholesteryl esters were scraped from the plates and methylated using BF3/methanol as previously described (Morrison and Smith 1964). Extracted methylated fatty acids were analyzed by an Agilent 7890A gas chromatograph equipped with flame ionization detectors, capillary column (SP2380, 0.25 mm × 30 m, 0.25 µm film, Supelco, Bellefonte, PA) with helium used as a carrier gas. The oven temperature was programmed from 160°C to 230°C at 4°C min^{-1}. Fatty acid methyl esters were identified by comparing the retention times to known standards. Inclusion of lipid standards with odd chain fatty acids permitted quantitation of the amount of lipid in the sample. Dipentadecanoyl phosphatidylcholine (C15:0), diheptadecanoin (C17:0), trieicosenoin (C20:1), and cholesteryl eicosenoate (C20:1) were used as standards.

Statistical Analysis

Data were analyzed by analysis of variance using Prism 6 analysis software (GraphPad Software, La Jolla, CA) with significance determined at $P < 0.05$.

RESULTS

Growth of Enterobacter cloacae Strains JD6301 and JD8715 in Conditions that Mimic those Encountered in Vivo

The bacterial strains *Enterobacter cloacae* JD6301 and JD8715 were recently characterized as oleaginous bacteria (Donaldson et al., 2014, Wilson et al., 2014). To determine if JD6301 and JD8715 have the capability of surviving within the mammalian GIT, the viability of these strains in acidic conditions and in the presence of bile was tested. Figure 1 indicates that both strains were able to grow in media supplemented with 5% porcine bile extract. Additionally, both JD6301 and JD8715 were able to survive acidic conditions to pH 4.0 (data not shown). Together, these data suggest that JD6301 and JD8715 can survive conditions encountered within the mammalian GI tract.

Alterations in the Serum Profiles in Pigs provided JD6301 or JD8715

Pigs were provided *Enterobacter cloacae* strains JD6301 or JD8715 or PBS via oral gavage daily for 6 d. There were no physical signs of differences observed between the groups, indicating that the *Enterobacter cloacae* strains did not impose a health risk to these animals. Serum was analyzed for triglycerides (TAG), NEFA, and

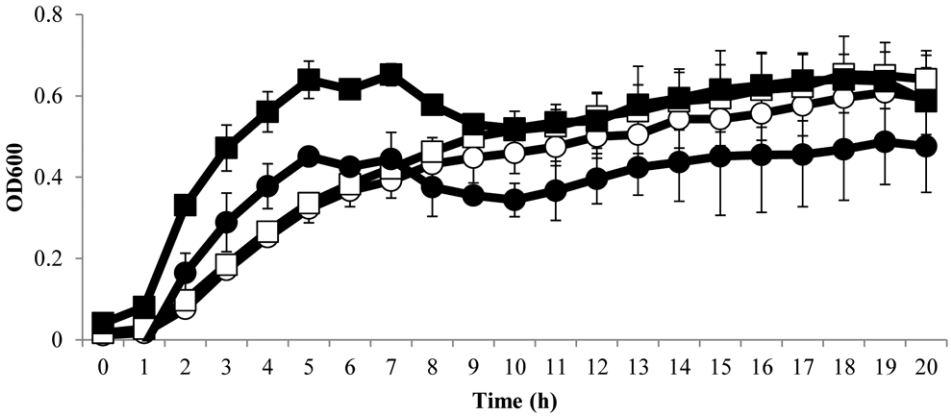

Figure 1. Growth of JD6301 and JD8715 in nutrient broth with porcine bile extract. Overnight cultures were diluted 1:100 in fresh nutrient broth (NB) or NB supplemented with 5% porcine bile extract and OD_{600} readings were measured hourly. JD6301 (O); JD8715 (□); JD6301 with 5% porcine bile extract (●); and JD8715 with 5% porcine bile extract (υ). Graph represents the average of 3 independent replicates. Error bars represent the standard deviation.

glycerol throughout the study. Serum TAG in pigs provided either JD6301 or JD8715 increased in comparison to PBS controls within 72 h ($P < 0.05$, Fig. 2 and Table 1). The serum concentrations of TAG were not significantly different between pigs provided JD6301 in comparison to those provided JD8715 except at 42 h after the initial dose. At this time point, serum concentrations increased in pigs provided JD6301 ($P < 0.001$).

The composition of serum fatty acids in pigs provided JD6301 or JD8715 were analyzed at 54h and 78h post the initial dose (Table 2). There was very little change in the composition of the serum fatty acids for pigs provided *Enterobacter cloacae*. A significant increase was observed for pigs provided JD6301 in palmitic acid (16:0). A decrease was observed in 18:2 and 18:3w3 in JD6301, but this effect was only significant at 54h. A slight increase in stearic acids (18:0) was observed for both sets of pigs, though this increase was not significant (Table 2).

Serum concentrations of NEFA also increased in pigs provided either JD6301 or JD8715 within 60 h after the initial supplementation (Table 3). Interestingly, this increase was detected earlier than the increase in TAG (60 h vs. 72 h). Serum concentrations of glycerol were also monitored during the study (Table 4). The concentrations of glycerol did not significantly change between pigs provided either JD6301 or JD8715 in comparison to pigs provided PBS only, the only exception being noted at 18h post the initial dose of JD6301 (Table 4). The se-

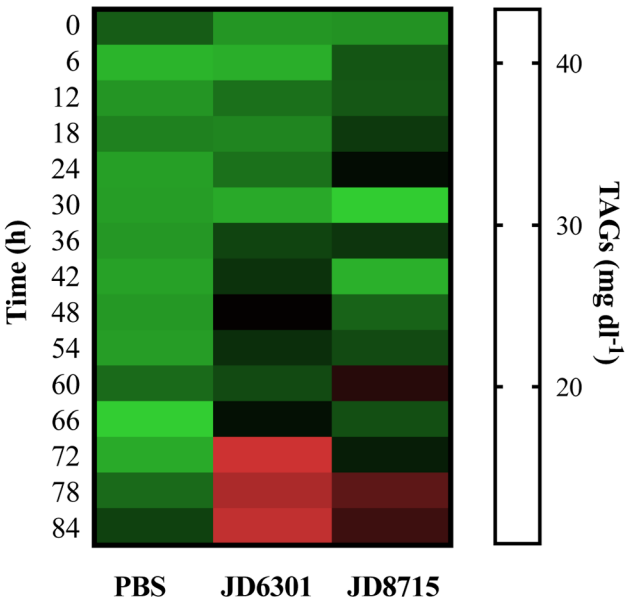

Figure 2. Heat map indicating concentration of serum triglycerides in pigs provided PBS, JD6301, or JD8715. Pigs were provided a 2 mL dose containing PBS, or a 1×10^9 CFU kg^{-1} of *Enterobacter cloacae* JD6301 or JD8715 daily by oral gavage ($n = 12$ per group). Serum was collected every 6 h and analyzed for TAG concentrations. Heat map represents the average concentration of each treatment group (mg dl^{-1}) in relation to time.

Table 1. Serum triglycerides from pigs provided a daily dose of phosphate buffered saline (PBS), *Enterobacter cloacae* strain JD6301, or JD8715

	TAG[1] (mg dl^{-1})			*P*-value		
Time (h)	PBS	JD6301	JD8715	PBS vs. JD6301	PBS vs. JD8715	JD6301 vs. JD8715
0	19.37	14.68	14.97	0.49	0.53	0.94
6	12.28	12.75	19.92	0.88	0.18	0.19
12	14.73	17.71	19.72	0.45	0.30	0.61
18	16.35	16.05	22.19	0.91	0.17	0.09
24	13.93	17.69	25.78	0.47	0.13	0.36
30	14.12	13.12	10.35	0.77	0.20	0.36
36	14.56	21.29	22.49	0.31	0.03	0.86
42	13.77	22.71	12.55	0.03	0.71	< 0.01
48	14.53	27.06	18.77	0.22	0.53	0.44
54	14.15	23.03	20.76	0.24	0.27	0.79
60	18.21	20.81	29.93	0.63	0.18	0.329
66	10.28	25.48	20.40	0.13	0.15	0.61
72	13.07	43.33	24.46	0.03	0.04	0.15
78	18.20	40.55	34.30	0.07	0.00	0.58
84	21.56	42.35	31.72	0.05	0.04	0.26

[1]TAG = triglycerides.

Table 2. Serum fatty acids (%) from pigs provided a daily dose of phosphate buffered saline (PBS), *Enterobacter cloacae* strain JD6301, or JD8715

	PBS			JD6301			JD8715			*P*-value					
Fatty acid	0 h	54 h	78 h	0 h	54 h	78 h	0 h	54 h	78 h	PBS0 vs. 54 h	PBS0 vs. 78 h	JD63010 vs. 54 h	JD63010 vs. 78 h	JD87150 vs. 54 h	JD87150 vs. 78 h
14:0	1.1	1.1	0.8	0.8	0.9	0.8	0.9	1.0	0.9	0.82	0.16	0.54	0.51	0.77	0.90
16:0	27.3	26.3	25.5	26.1	27.9	26.9	27.4	26.5	26.5	0.57	0.15	0.05	0.30	0.80	0.74
16:1	1.3	1.2	1.3	1.1	1.4	1.2	1.5	1.3	1.1	0.36	0.96	0.37	0.61	0.98	0.22
18:0	8.2	7.0	8.3	7.5	8.7	8.9	7.6	8.7	8.4	0.27	0.93	0.24	0.25	0.69	0.48
18:1w9	34.2	33.2	31.4	32.0	34.2	34.7	32.6	31.9	29.5	0.32	0.21	0.22	0.14	0.75	0.14
18:1w7	1.6	1.5	1.6	1.5	1.5	1.5	1.4	1.5	1.4	0.41	0.83	0.74	0.68	0.72	0.79
18:2	23.7	26.1	26.4	27.4	23.1	23.7	25.2	24.8	26.5	0.31	0.24	0.05	0.13	0.91	0.47
18:3w6	0.1	0.4	0.3	0.2	0.2	0.1	0.4	0.3	0.4	0.06	0.30	0.86	0.76	0.78	0.64
18:3w3	1.0	1.1	1.1	1.2	1.0	0.7	1.1	0.8	1.1	0.44	0.56	0.05	0.26	0.41	0.56

Table 3. NEFA serum concentrations (mEq l^{-1}) from pigs provided a daily dose of phosphate buffered saline (PBS), *Enterobacter cloacae* strain JD6301, or JD8715

	NEFA[1] (mEq l^{-1})			*P*-value	
Time (h)	PBS	JD6301	JD8715	JD6301 vs. PBS	JD8715 vs. PBS
0	0.16	0.13	0.13	0.17	0.41
6	0.23	0.24	0.25	0.86	0.74
12	0.12	0.16	0.15	0.20	0.18
18	0.13	0.23	0.32	0.22	0.05
24	0.15	0.15	0.14	0.95	0.74
30	0.14	0.24	0.15	0.17	0.90
36	0.18	0.16	0.26	0.58	0.19
42	0.15	0.11	0.34	0.29	0.10
48	0.24	0.16	0.34	0.51	0.53
54	0.28	0.18	0.46	0.22	0.20
60	0.17	0.40	0.54	0.03	< 0.01
66	0.25	0.48	0.61	0.04	0.02
72	0.52	0.33	0.44	0.09	0.44
78	0.27	0.57	0.41	0.04	0.22
84	0.29	0.35	0.54	0.52	0.03

[1]NEFA = non-esterified fatty acids.

Table 5. Glucose serum concentrations in pigs provided phosphate buffered saline (PBS), *Enterobacter cloacae* strain JD6301, or JD8715

	Glucose			*P*-value	
Time (h)	PBS	JD6301	JD8715	JD6301 vs. PBS	JD8715 vs. PBS
0	102.01	104.25	115.40	0.53	0.24
6	99.44	114.65	111.50	0.01	0.07
12	98.65	96.27	95.43	0.80	0.58
18	90.08	99.32	97.54	0.40	0.53
24	93.18	104.54	101.14	0.12	0.34
30	84.99	101.03	92.74	0.16	0.11
36	80.06	90.57	86.65	0.35	0.58
42	89.75	81.31	89.77	0.13	1.00
48	87.02	83.98	89.25	0.54	0.85
54	89.44	82.68	89.41	0.28	1.00
60	83.48	83.61	78.53	0.99	0.47
66	102.29	100.13	113.73	0.76	0.59
72	104.27	114.17	97.39	0.17	0.31
78	88.41	104.30	99.17	0.15	0.21
84	103.44	98.70	98.24	0.42	0.48
96	106.30	100.82	105.19	0.84	0.96

rum concentration of glucose was not significantly different, except at 6 h post the initial dose (Table 5).

Intestinal and Fecal Populations of E. cloacae

Fecal samples were collected from each pig daily and analyzed for the presence of either JD6301 or JD8715. The greatest populations of *Enterobacter cloacae* were observed 48 h after the initial dosage (Fig. 3). The presence of either *Enterobacter* strain was minimal in the feces past this time point.

To determine whether the organisms were colonizing the gastrointestinal tract, the intestinal presence of JD6301 and JD8715 was analyzed in the cecum, jejunum, and ileum from pigs on d 6. The greatest populations of *E. cloacae* were present within the ileum and the jejunum (Fig. 4), which may indicate that the small intestine is the preferred site of colonization of JD6301 or JD8715 when provided via oral supplementation. This result was confirmed through enrichment cultures (data not shown).

Table 4. Serum glycerol (mg l^{-1}) from pigs provided phosphate buffered saline (PBS), *Enterobacter cloacae* strain JD6301, or JD8715

	Glycerol (mg l^{-1})			*P*-value	
Time (h)	PBS	JD6301	JD8715	JD6301 vs. PBS	JD8715 vs. PBS
0	0.03	0.05	0.03	0.32	0.90
6	0.04	0.06	0.07	0.32	0.14
12	0.05	0.04	0.05	0.28	0.58
18	0.03	0.05	0.04	0.09	0.36
24	0.04	0.05	0.04	0.15	0.72
30	0.02	0.04	0.04	0.01	0.33
36	0.03	0.04	0.04	0.55	0.54
42	0.04	0.07	0.04	0.16	0.82
48	0.05	0.06	0.04	0.54	0.57
54	0.04	0.03	0.07	0.15	0.25
60	0.10	0.07	0.09	0.59	0.95
66	0.06	0.10	0.07	0.56	0.88
72	0.12	0.07	0.06	0.38	0.27
78	0.07	0.08	0.06	0.73	0.76
84	0.09	0.07	0.06	0.46	0.53

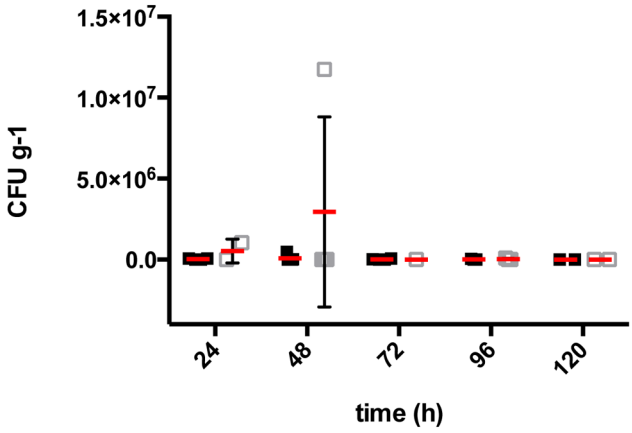

Figure 3. Presence of *Enterobacter cloacae* strains JD6301 or JD8715 in feces. Fecal samples were collected daily following oral supplementation with JD6301 (□) or JD8715 (υ). Individual values are plotted for pigs with positive feces, with means represented in red. Error bars represent the standard deviation.

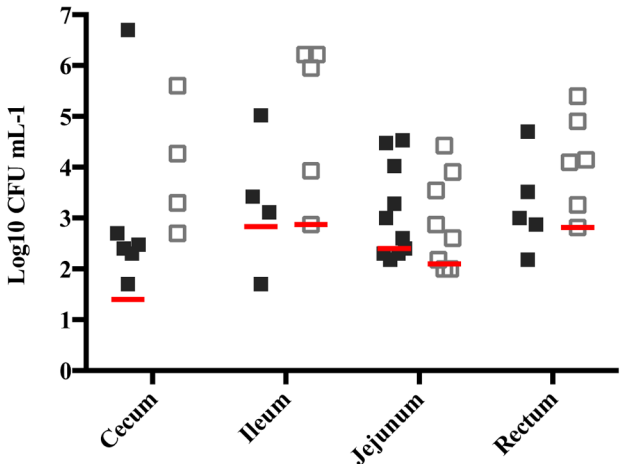

Figure 4. Intestinal populations of *Enterobacter* strains JD6301 or JD8715. The cecum, ileum, jejunum, and rectum of pigs provided JD6301 (□) or JD8715 (υ) were analyzed after 6 d of oral supplementation. Individual values (log_{10} CFU mL^{-1}) for pigs positive for JD6301 or JD8715 are plotted, with means represented as red lines.

DISCUSSSION

Lack of sufficient energy reserves can impact the immune response. For instance, during times of weaning, pigs are more prone to enteric infections due to a lack of sufficient energy reserves and difficulty in initiating feeding (Metz and Gonyou 1990). With the rise in antibiotic resistant microorganisms and restrictions on the use of antibiotics increasing in the livestock industry, it is imperative to identify alternative sources to combat infections. Supplementing feed with triglycerides has been shown to improve energy supplies and decrease mortality associated with neonatal pigs (Jean and Chiang 1999). Therefore, this study tested whether the *Enterobacter cloacae* strains JD6301 and JD8715 could be used as potential probiotics that provide an additional source of lipids to pigs, thus improving the availability of energy.

Weanling pigs were provided JD6301, JD8715, or PBS via oral gavage daily and serum was analyzed every 6 h throughout the study. Several components of the serum were found to be altered following administration of these *Enterobacter* strains. TAG were found to significantly increase in pigs provided either JD6301 or JD8715 in comparison to those provided PBS. Over the 84h period assessed, JD6301 increased serum TAG from 14.68mg dl^{-1} to 42.35 mg dl^{-1} (p = 0.01) and JD8715 increased serum TAG from 14.97 mg dl^{-1} to 31.72 mg dl^{-1} (p = 0.002). There was not a significant increase in TAG observed in pigs provided PBS. This indicated that the increase was due to the *Enterobacter cloacae* strains provided. An increase in the serum concentration of non-esterified fatty acids increased in pigs provided either strain of *Enterobacter cloacae.* However, the increase was greater among pigs provided JD8715 (threefold increase in comparison to 1.7-fold increase). This could indicate the increase of the fatty acids released by the bacteria or the activity of lipoprotein lipase against the provided TAG. Regardless, the increase in the prevalence of serum fatty acids suggests that lipids released by the JD8715 strain may be more readily accessible by weanling pigs. The lack of a significant alteration in glucose concentrations indicates that though the TAG concentrations increased, there was not an increased energy demand in the animals.

To further examine the increase in TAG observed in pigs provided JD6301 or JD8715, serum fatty acid profiles were examined in comparison to PBS control pigs. A slight alteration in the profile was detected, though the change was only significant for pigs provided JD6301 at 54 h of analysis. A change in the profile was expected, though, as serum fatty acid profiles have been previously reported to be altered in response to alterations in fats provided to pigs (Vicente et al., 2013). The lack of a significant alteration in the fatty acid composition profile suggests that the supplementation of with either strain of *E. cloacae* does not alter lipid metabolism.

There was not a significant alteration in the serum concentration of glucose except at 6 h post the initial dose of JD6301 or JD8715. This suggests that providing *E. cloacae* did not increase the energy demand of the pigs. Nevertheless, the increase observed in TAG and NEFA is an important physiological factor of the pig that needs to be explored further.

Populations of *E. cloacae* were predominately located in the ileum and jejunum. The jejunum contains lipase activity, which digests TAG to facility absorption of fatty acids. The presence of this unique bacterium in this site is ideal, as this location would allow for maximum absorption of lipids across the enterocytes (Iqbal and Hussain 2009).

Together, these data suggest that *E. cloacae* strains JD6301 or JD8715 can be used as a novel probiotic to increase the availability of energy to animals. The data also suggest that providing either strain of *E. cloacae* did not impact lipid metabolism or increase energy demand. The increase in TAG and NEFA indicate that the pigs were able to utilize the lipids provided by the bacteria. This is further supported by the presence of these bacteria in the ileum and jejunum. Though this research identifies a potential use of bacteria to provide lipids to weanling pigs, further research is still needed to determine if this mechanism of providing additional energy can improve the metabolic response of the animal in the presence of an enteric infection or during stress. Additionally, impacts that these bacteria have the microbial community within the intestine is needed.

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