Supplementary materials

Supplementary Table 1. 16S and 23S rRNA gene-targeted primers used in this study

Target bacteria*	Primer	Sequence (5' - 3')	Ref
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	5
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA	
Clostridium leptum subgroup	sg-Clept-F	GCACAAGCAGTGGAGT	6
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA	
Bacteroides fragilis group	g-Bfra-F2	AYAGCCTTTCGAAAGRAAGAT	7
	g-Bfra-R	CCAGTATCAACTGCAATTTTA	5
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	5
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA	
Atopobium cluster	g-Atopo-F	GGGTTGAGAGACCGACC	6
	g-Atopo-R	CGGRGCTTCTTCTGCAGG	
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	5
	g-Prevo-R	GGTCGGGTTGCAGACC	
Clostridium perfringens	s-Clper-F	GGGGGTTTCAACACCTCC	2
	ClPER-R	GCAAGGGATGTCAAGTGT	4
Lactobacillus gasseri subgroup	sg-Lgas-F	GATGCATAGCCGAGTTGAGAGACTGAT	2
	sg-Lgas-R	TAAAGGCCAGTTACTACCTCTATCC	
Lactobacillus brevis	s-Lbre-F	ATTTTGTTTGAAAGGTGGCTTCGG	2
	s-Lbre-R	ACCCTTGAACAGTTACTCTCAAAGG	
Lactobacillus casei subgroup	sg-Lcas-F	ACCGCATGGTTCTTGGC	2
	sg-Lcas-R	CCGACAACAGTTACTCTGCC	
Lactobacillus fermentum	LFer-1	CCTGATTGATTTTGGTCGCCAAC	2
	LFer-2	ACGTATGAACAGTTACTCTCATACGT	
Lactobacillus fructivorans	s-Lfru-F	TGCGCCTAATGATAGTTGA	2
	s-Lfru-R	GATACCGTCGCGACGTGAG	
Lactobacillus plantarum subgroup	sg-Lpla-F	CTCTGGTATTGATTGGTGCTTGCAT	2
	sg-Lpla-R	GTTCGCCACTCACTCAAATGTAAA	
Lactobacillus reuteri subgroup	sg-Lreu-F	GAACGCAYTGGCCCAA	2
	sg-Lreu-R	TCCATTGTGGCCGATCAGT	
Lactobacillus ruminis subgroup	sg-Lrum-F	CACCGAATGCTTGCAYTCACC	2
	sg-Lrum-R	GCCGCGGGTCCATCCAAAA	
Lactobacillus sakei subgroup	sg-Lsak-F	CATAAAACCTAMCACCGCATGG	2
	sg-Lsak-R	TCAGTTACTATCAGATACRTTCTTCTC	
Enterobacteriaceae	En-lsu-3F	TGCCGTAACTTCGGGAGAAGGCA	1
	En-lsu-3'R	TCAAGGACCAGTGTTCAGTGTC	
Enterococcus	g-Encoc-F	ATCAGAGGGGGATAACACTT	2
	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC	
Staphylococcus	g-Staph-F	TTTGGGCTACACACGTGCTACAATGGACAA	2
	g-Staph-R	AACAACTTTATGGGATTTGCWTGA	
Streptococcus	g-Str-F	AGCTTAGAAGCAGCTATTCATTC	3
	g-Str-R	GGATACACCTTTCGGTCTCTC	
Pseudomonas	PSD7F	CAAAACTACTGAGCTAGAGTACG	1
	PSD7R	TAAGATCTCAAGGATCCCAACGGCT	

*Specific primer sets were developed using 16S rDNA sequences, except for En-lsu-3F/3'R, and g-Str-F/R, which targeted 23S rDNA.

References

1. Matsuda K, Tsuji H, Asahara T, et al. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. *Appl Environ Microbiol* 2007; 73: 32-39.
2. Matsuda K, Tsuji H, Asahara T, et al. Establishment of an analytical system for the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of multicopy rRNA molecules. *Appl Environ Microbiol* 2009; 75: 1961-1969.
3. Sakaguchi S, Saito M, Tsuji H, et al. Bacterial rRNA-targeted reverse transcription-PCR used to identify pathogens responsible for fever with neutropenia. *J Clin Microbiol* 2010; 48: 1624-1628.
4. Kikuchi E, Miyamoto Y, Narushima S, et al. Design of species specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. *Microbiol Immunol* 2002; 46: 353–358.
5. Matsuki T, Watanabe K, Fujimoto J, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol* 2002; 68: 5445-5451.
6. Matsuki T, Watanabe K, Fujimoto J, et al. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol* 2004; 70: 7220-7228.
7. Matsuki T. Development of quantitative PCR detection method with 16S rRNA gene-targeted genus- and species-specific primers for the analysis of human intestinal microflora and its application. *Nihon Saikingaku Zasshi* 2007; 62: 255-261. [Article in Japanese]

Supplementary Table 2. Fecal bacterial counts and detection rates before middle cerebral artery occlusion in *db/+*, *db/db*, and polymyxin B-treated *db/db* mice (n = 5 per group).

	db/+		*db/db*		polymyxin B-treated *db/db*	
	Bacterial count (log_{10} cells/g)	Detection rate (%)	Bacterial count (log_{10} cells/g)	Detection rate (%)	Bacterial count (log_{10} cells/g)	Detection rate (%)
Total bacteria	9.3 ± 0.3		9.5 ± 0.3		9.5 ± 0.1	
Obligate anaerobes						
Clostridium coccoides group	8.3 ± 0.4	100	8.8 ± 0.4	80	8.5 ± 0.2	100
Clostridium leptum subgroup	8.5 ± 0.4	100	8.8 ± 0.2	80	8.7 ± 0.2	100
Bacteroides fragilis group	8.4 ± 0.4	100	8.6 ± 0.1	100	8.7 ± 0.3	100
Bifidobacterium	8.2 ± 1.0	100	<5.0	0	8.6 ± 0.6	40
Atopobium cluster	5.5 ± 0.7	60	6.6 ± 0.6	100	7.1 ± 0.5	100
Prevotella	<5.0	0	<5.0	0	<5.0	0
Clostridium perfringens	5.2 ± 0.1	60	4.4	20	4.4 ± 0	40
Facultative anaerobes						
Total *Lactobacillus*	8.9 ± 0.3		9.0 ± 0.3		9.2 ± 0.1	
Lactobacillus gasseri subgroup	8.5 ± 0.1	80	8.5 ± 0.4	40	8.9 ± 0.1	60
Lactobacillus brevis	2.7 ± 0.2	100	3.5 ± 0.2	100	3.3 ± 0.2	100
Lactobacillus casei subgroup	<3.0	0	<3.0	0	3.1	20
Lactobacillus fermentum	<4.0	0	<4.0	0	<4.0	0
Lactobacillus fructivorans	<2.4	0	<2.4	0	<2.4	0
Lactobacillus plantarum subgroup	3.5 ± 0.2	100	3.7 ± 0.3	100	3.5 ± 0.1	100
Lactobacillus reuteri subgroup	8.6 ± 0.4	100	8.8 ± 0.4	100	9.0 ± 0.2	100
Lactobacillus ruminis subgroup	7.0 ± 0.4	100	7.8 ± 0.3	100	7.9 ± 0.6	100
Lactobacillus sakei subgroup	3.7	20	2.4	20	<2.3	0
Enterobacteriaceae	5.1 ± 0.3	60	7.5 ± 0.6	100	5.5 ± 0.7	100
Enterococcus	6.6 ± 0.3	100	8.7 ± 0.5	100	7.5 ± 0.5	100
Staphylococcus	5.7 ± 0.4	100	5.7 ± 0.2	100	5.6 ± 0.3	100
Streptococcus	7.1 ± 0.7	100	6.3 ± 0.5	80	7.4 ± 0.2	100
Aerobes						
Pseudomonas	<3.0	0	<3.0	0	<3.0	0

Data are presented as mean ± SD.

Supplementary Table 3. Comparisons of plasma lipopolysaccharide (LPS) levels before and after middle cerebral artery occlusion (MCAO) in *db/+*, *db/db*, and polymyxin B-treated *db/db* mice (n = 6-9 per group).

	Before MCAO	After MCAO	
	LPS (EU/mL)		P value
db/+	2.1 ± 1.1	6.7 ± 2.3	<0.01
db/db	6.5 ± 0.9	19.6 ± 0.9	<0.001
polymyxin B-treated *db/db*	3.8 ± 1.1	10.1 ± 2.9	<0.001

Data are presented as mean ± SD.

Supplementary Table 4. Comparisons of intestinal permeability assessed by quantitative analysis of fluorescein isothiocyanate (FITC)-dextran translocation before and after middle cerebral artery occlusion (MCAO) in *db/+*, *db/db*, and polymyxin B-treated *db/db* mice (n = 5 per group).

	Before MCAO	After MCAO	
	FITC-dextran in blood (ng/mL)		P value
db/+	12.2 ± 9.2	27.4 ± 8.2	<0.05
db/db	32.7 ± 7.1	108.8 ± 7.1	<0.001
polymyxin B-treated *db/db*	17.6 ± 4.3	46.5 ± 4.3	<0.01

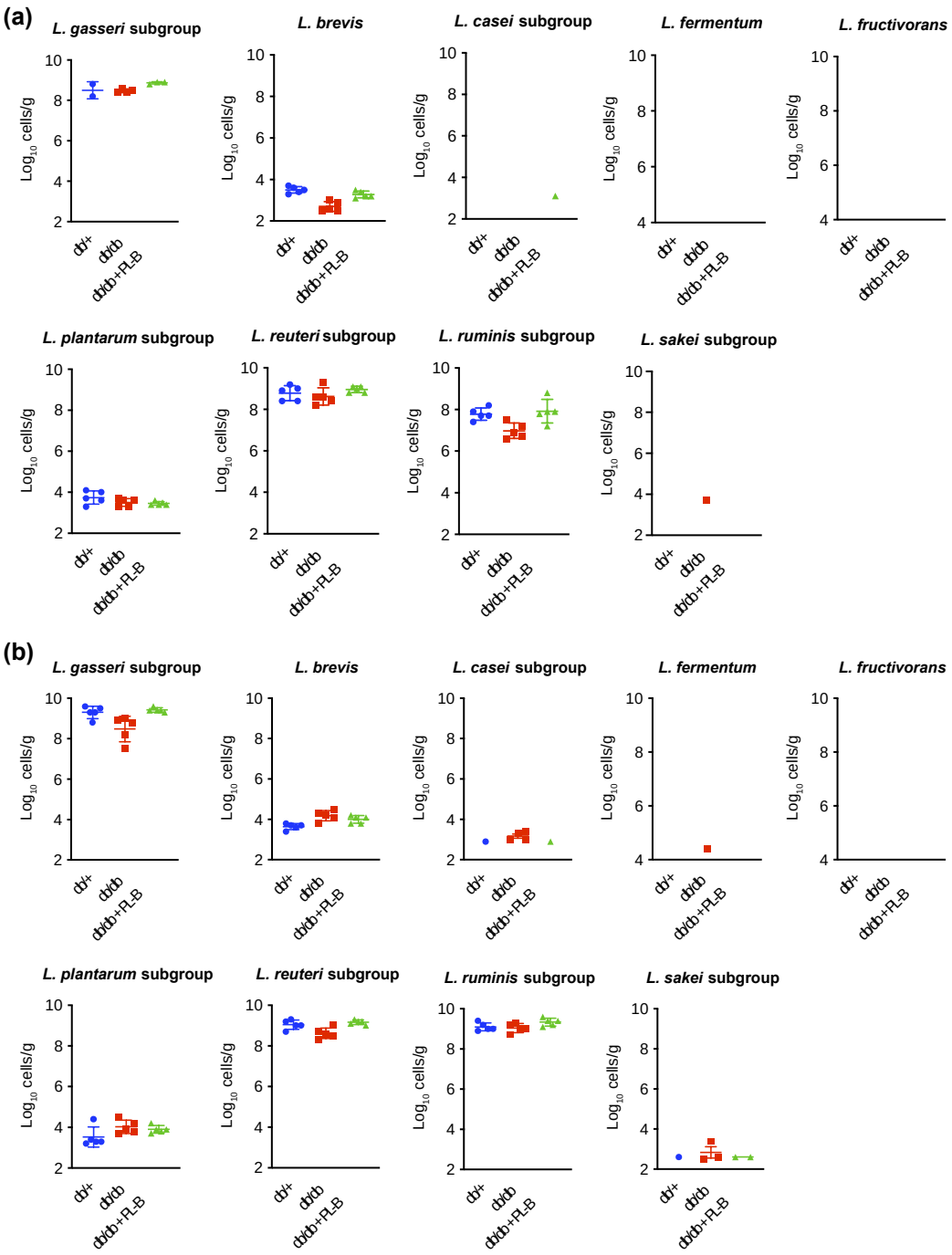
Data are presented as mean ± SD.

Supplementary Table 5. Fecal bacterial counts and detection rates after middle cerebral artery occlusion in *db/+*, *db/db*, and polymyxin B-treated *db/db* mice (n = 5 per group).

	db/+		*db/db*		polymyxin B-treated *db/db*	
	Bacterial count (log_{10} cells/g)	Detection rate (%)	Bacterial count (log_{10} cells/g)	Detection rate (%)	Bacterial count (log_{10} cells/g)	Detection rate (%)
Total bacteria	9.8 ± 0.1		9.7 ± 0.1		10.0 ± 0.2	
Obligate anaerobes						
Clostridium coccoides group	9.0 ± 0.1	100	8.9 ± 0.2	100	9.1 ± 0.3	100
Clostridium leptum subgroup	8.9 ± 0.2	100	8.9 ± 0.3	100	8.9 ± 0.4	100
Bacteroides fragilis group	8.6 ± 0.2	100	8.9 ± 0.2	100	8.7 ± 0.3	100
Bifidobacterium	<4.9	0	<4.9	0	<4.9	0
Atopobium cluster	<5.0	0	<5.0	0	<5.0	0
Prevotella	<5.0	0	<5.0	0	<5.0	0
Clostridium perfringens	<2.3	0	<2.3	0	<2.3	0
Facultative anaerobes						
Total *Lactobacillus*	9.7 ± 0.2		9.3 ± 0.1		9.8 ± 0.1	
Lactobacillus gasseri subgroup	9.3 ± 0.3	100	8.5 ± 0.6	100	9.4 ± 0.1	100
Lactobacillus brevis	3.6 ± 0.2	100	4.2 ± 0.3	100	4.0 ± 0.2	100
Lactobacillus casei subgroup	2.9	20	3.2 ± 0.2	80	2.9	20
Lactobacillus fermentum	<4.0	0	4.4	20	<4.0	0
Lactobacillus fructivorans	2.4 ± 0.1	40	<2.3	0	<2.3	0
Lactobacillus plantarum subgroup	3.5 ± 0.5	100	4.0 ± 0.3	100	3.9 ± 0.2	100
Lactobacillus reuteri subgroup	9.0 ± 0.2	100	8.6 ± 0.3	100	9.2 ± 0.1	100
Lactobacillus ruminis subgroup	9.1 ± 0.2	100	9.0 ± 0.2	100	9.3 ± 0.2	100
Lactobacillus sakei subgroup	2.6	20	2.8 ± 0.5	60	2.6 ± 0	40
Enterobacteriaceae	<4.0	0	5.1	20	6.1	20
Enterococcus	7.9 ± 0.2	100	7.9 ± 0.4	100	8.5 ± 0.3	100
Staphylococcus	5.6 ± 0.3	100	5.7 ± 0.4	100	5.0 ± 0.3	100
Streptococcus	7.4 ± 0.2	100	7.9 ± 0.4	100	7.8 ± 0.5	100
Aerobes						
Pseudomonas	<2.9	0	<2.9	0	<2.9	0

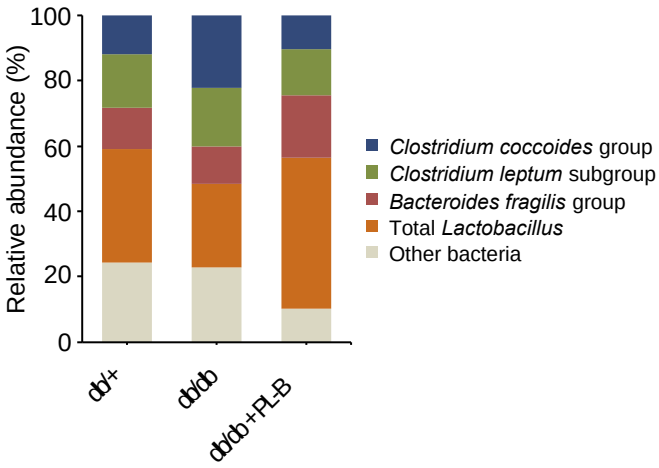
Data are presented as mean ± SD.

Supplementary Figure 1



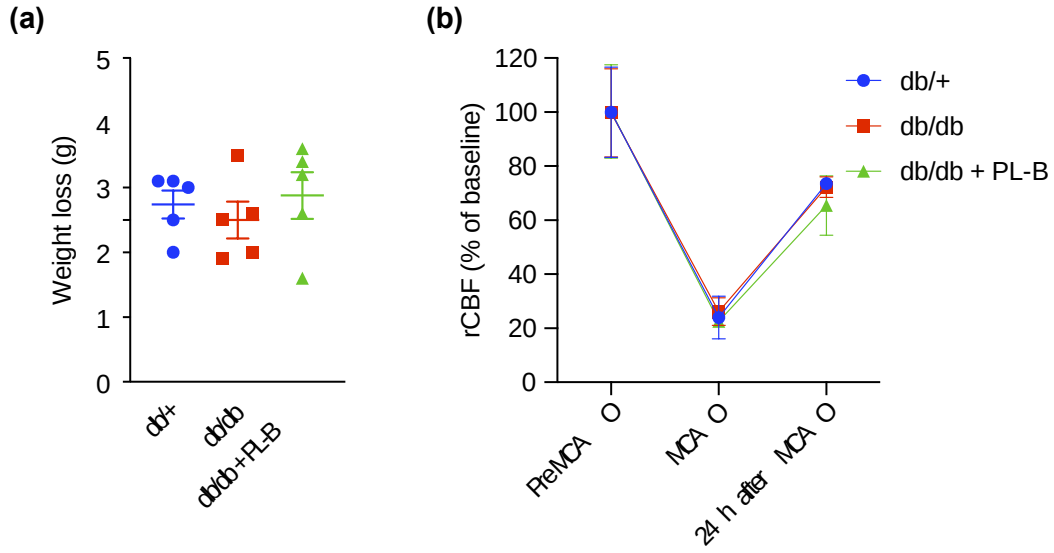
Supplementary Figure 1. Fecal *Lactobacillus* counts (a) before middle cerebral artery occlusion (MCAO) and (b) 24 h after MCAO analyzed by rRNA-targeted quantitative reverse transcription PCR in *db/+*, *db/db*, and polymyxin B (PL-B)-treated *db/db* mice (n = 5 per group). Bacterial counts below the threshold of detection were not plotted. Data are shown as mean ± SD.

Supplementary Figure 2



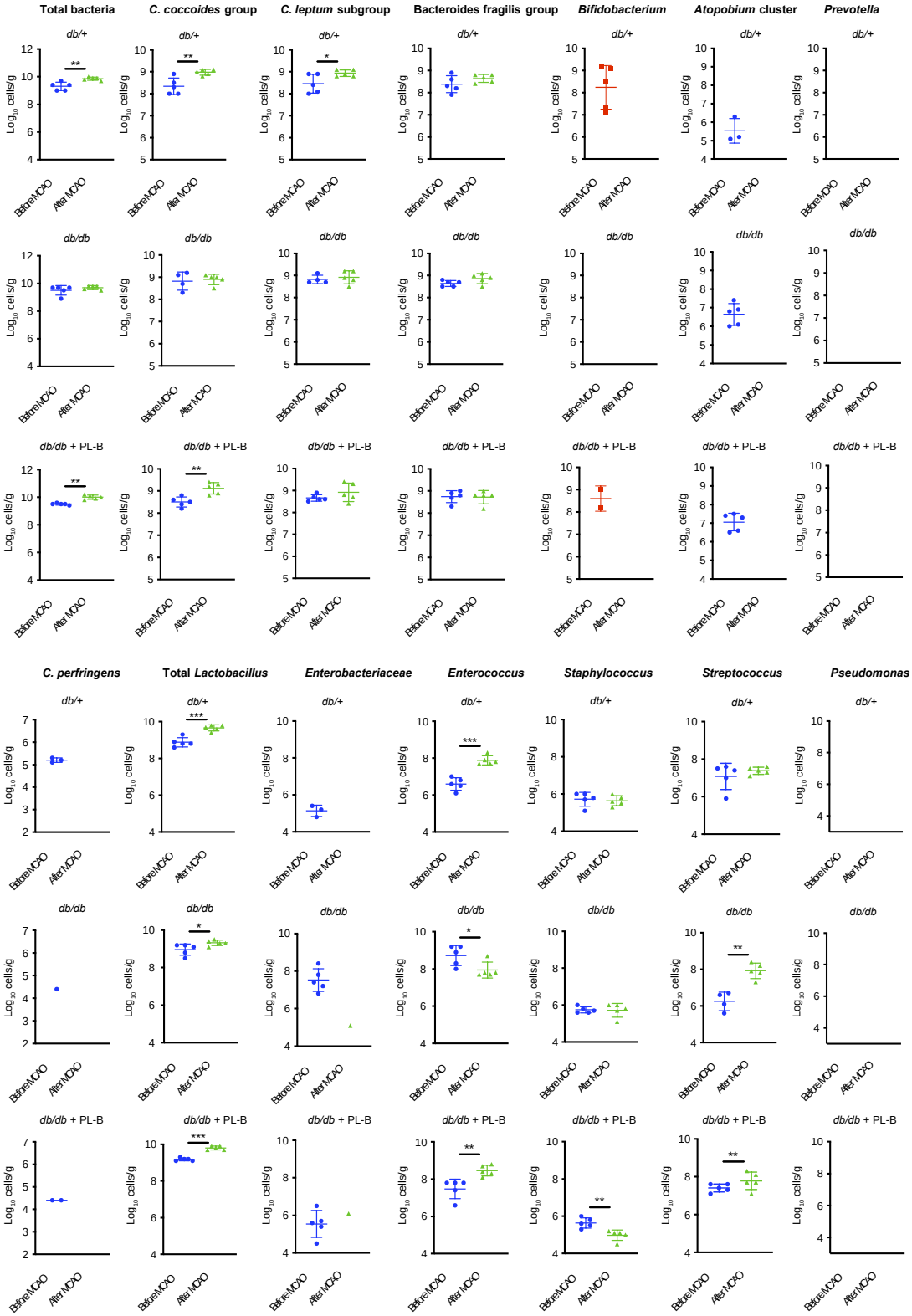
Supplementary Figure 2. Relative abundance of predominant bacteria before middle cerebral artery occlusion in *db/+*, *db/db*, and polymyxin B (PL-B)-treated *db/db* mice (n = 5 per group).

Supplementary Figure 3



Supplementary Figure 3. Physiological parameters. (a) Weight loss (baseline – 24 h after middle cerebral artery occlusion (MCAO)) (n = 5 per group) and (b) temporal changes in regional cerebral blood flow (rCBF) in ipsilateral hemispheres (n = 5 per group) in *db/+*, *db/db*, and polymyxin B (PL-B)-treated *db/db* mice. Data are shown as mean ± SD.

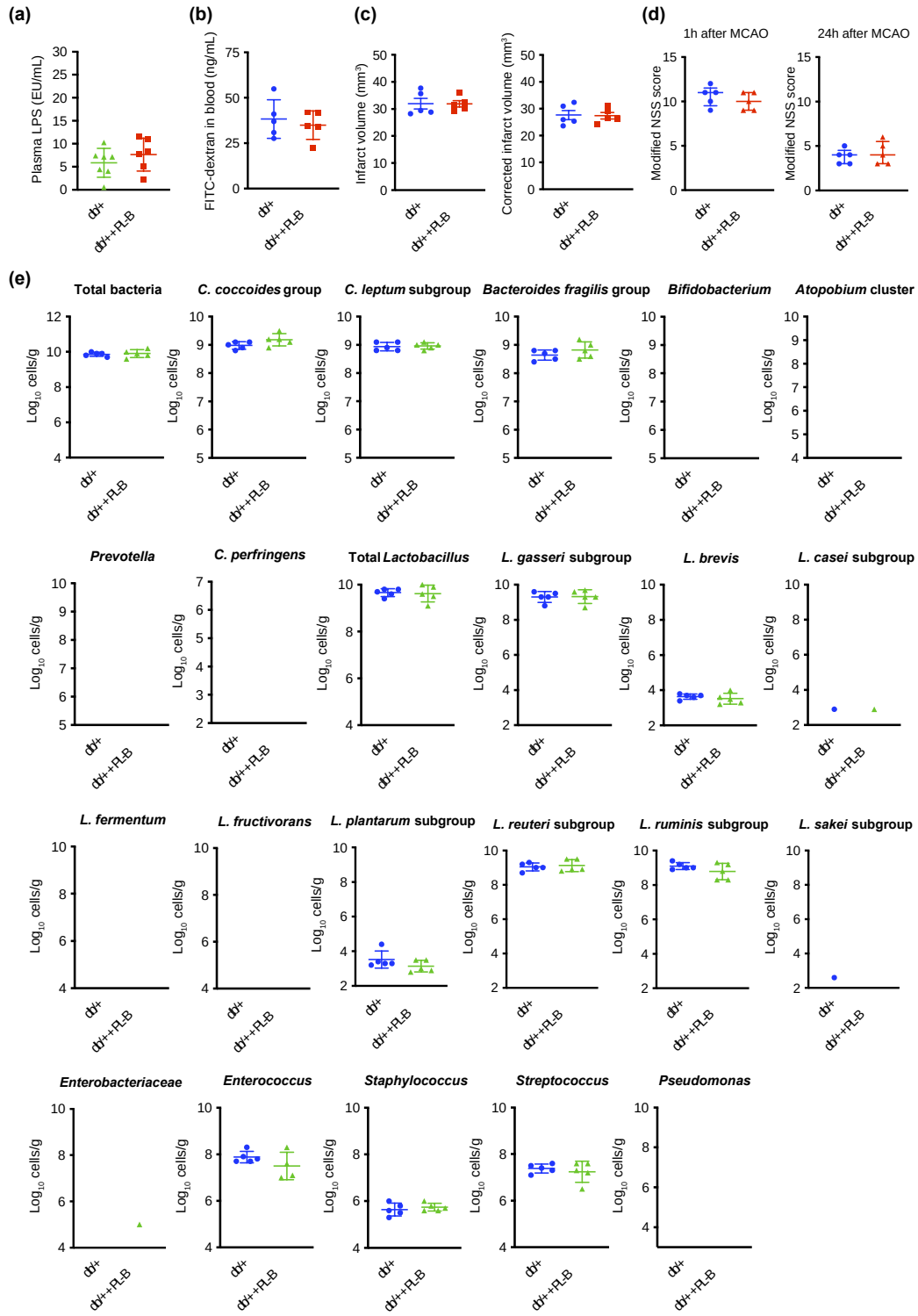
Supplementary Figure 4



Supplementary Figure 4. Comparisons of fecal bacterial counts before and 24 h after middle cerebral

artery occlusion (MCAO) analyzed by rRNA-targeted quantitative reverse transcription PCR in *db/+*, *db/db*, and polymyxin B (PL-B)-treated *db/db* mice (n = 5 per group). Bacterial counts below the threshold of detection were not plotted. Data are shown as mean ± SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

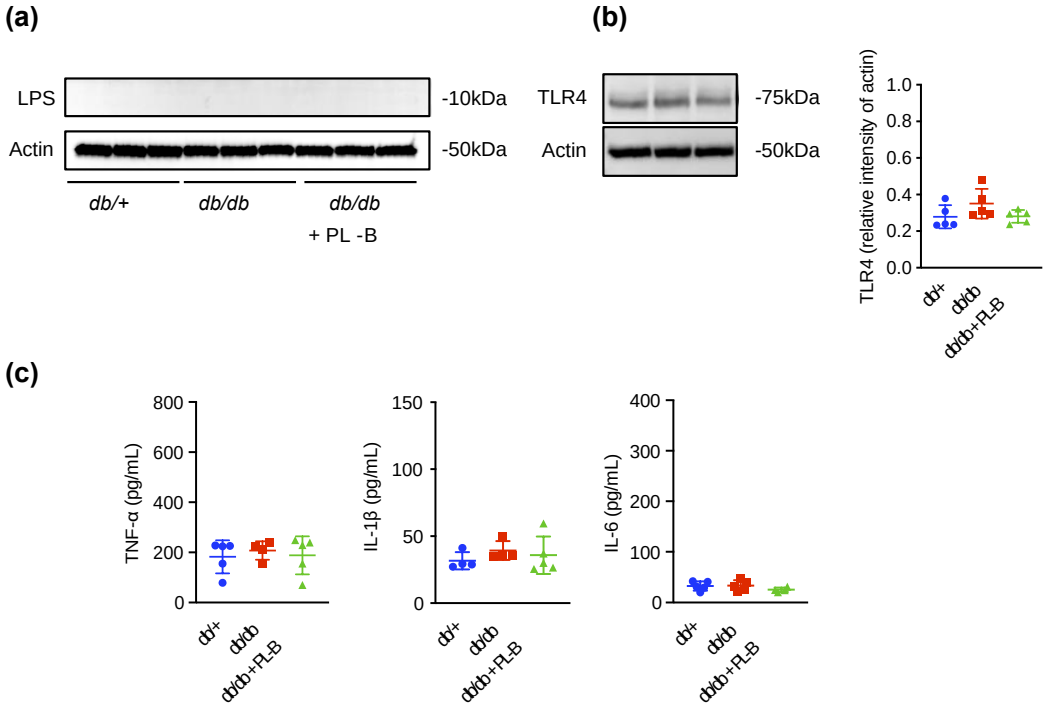
Supplementary Figure 5



Supplementary Figure 5. Effects of polymyxin B (PL-B) on stroke outcome in *db/+* mice. (a) Plasma lipopolysaccharide (LPS) levels 24 h after middle cerebral artery occlusion (MCAO) (n = 5 per group). (b) Intestinal permeability 3 h after MCAO (n = 5 per group). (c) Quantification of infarct volumes 24 h after

MCAO in *db/+* and polymyxin B-treated *db/+* mice. Total hemispheric infarct volumes were corrected for edema (n = 5 per group). Data are shown as mean ± SD. (d) Modified neurological severity scores (NSS) at 1 h and 24 h after MCAO in *db/+* and polymyxin B-treated *db/+* mice (n = 5 per group). Data are shown as median and interquartile range. (e) Fecal bacterial counts 24 h after MCAO analyzed by rRNA-targeted quantitative reverse transcription PCR in *db/+* and polymyxin B-treated *db/+* mice (n = 5 per group). Bacterial counts below the threshold of detection were not plotted. Data are shown as mean ± SD.

Supplementary Figure 6



Supplementary Figure 6. Expression of lipopolysaccharide (LPS), toll-like receptor 4 (TLR4), and inflammatory cytokines in non-ischemic hemispheres 24 h after middle cerebral artery occlusion. Western blot analyses of (a) LPS and (b) TLR4 expression. (c) Enzyme-linked immunoassay results for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. All data were analyzed in *db/+*, *db/db*, and polymyxin B (PL-B)-treated *db/db* mice sacrificed 24 h after middle cerebral artery occlusion (n = 4–5 per group). Data are shown as mean ± SD.