

High stocking density triggers stress-induced physiological changes and alters nasal and fecal microbiota in finishing pigs

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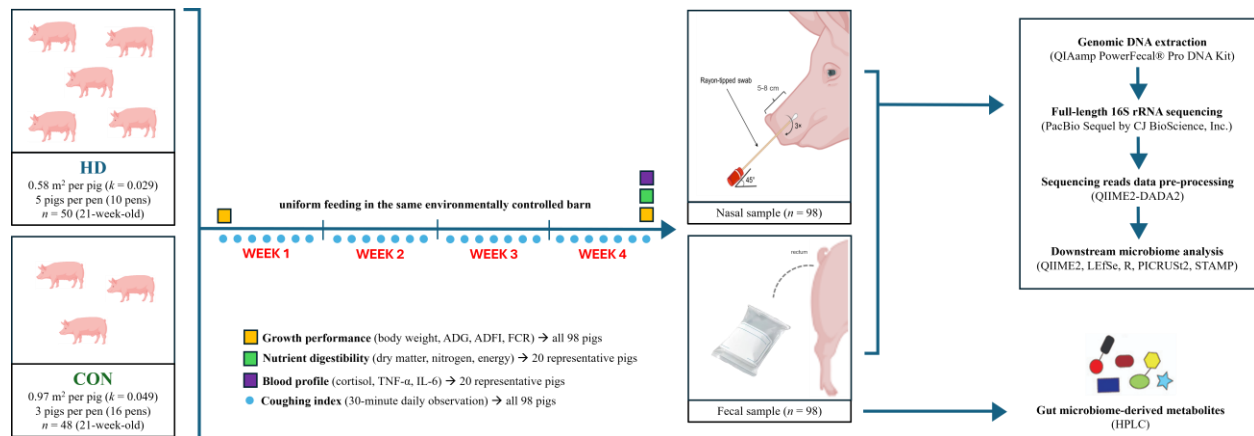
Supplementary Materials

Supplementary Table S1. Composition of finishing pig diet (as fed-basis).

Item	Basal diet
Ingredients (%)	
Corn	83.46
Soybean meal	11.70
Tallow	0.17
Molasses	2.00
MCP	0.80
Limestone	0.66
Lysine (78%)	0.36
Methionine (98%)	0.02
Threonine (98%)	0.11
Tryptophan (98%)	0.02
Salt	0.20
Mineral mix ¹	0.20
Vitamin mix ²	0.20
Choline (25%)	0.10
Total	100.00
Calculated value	
Crude protein, %	13.00
Metabolizable Energy, kcal/kg	3300
Calcium, %	0.52
Phosphorus, %	0.47
Lysine, %	0.84
Methionine, %	0.25
Threonine, %	0.56
Tryptophan, %	0.15
Crude Fat, %	3.25
Crude Fiber, %	2.11
Crude Ash, %	3.40

¹ Provided per kg diet: Fe, 100 mg as ferrous sulfate; Cu, 17 mg as copper sulfate; Mn, 17 mg as manganese oxide; I, 0.5 mg as potassium iodide; and Se, 0.3 mg as sodium selenite.

² Provided per kilograms of diet: vitamin A, 10,800 IU; vitamin D3, 4,000 IU; vitamin E, 40 IU; vitamin K3, 4 mg; vitamin B1, 6 mg; vitamin B2, 12 mg; vitamin B6, 6 mg; vitamin B12, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg.



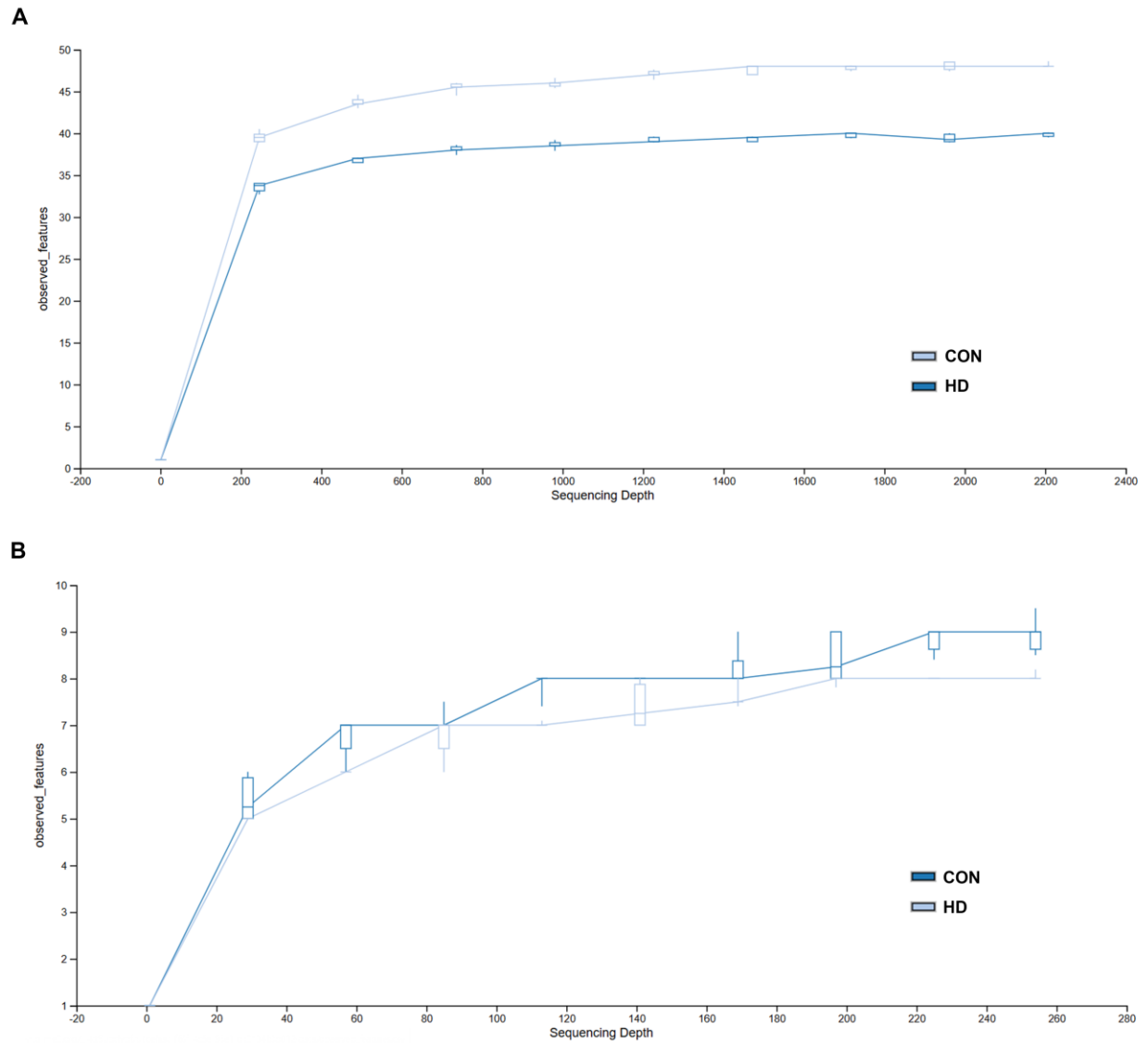
Supplementary Fig. S1. Experimental setup and sampling scheme of the stocking density experiment in finishing pigs.

Supplementary Table S2. Next-generation sequencing of nasal and fecal samples from finishing pigs.

Groups	Total raw reads	Valid reads ¹					
		Total	Minimum	Maximum	Average	Good's coverage (%)	Average length (bp)
Nasal							
CON	990,375	409,517	2,208	14,760	8,531.60	100	1,444.39
HD	1,091,826	404,358	3,287	14,123	8,087.16	100	
Fecal							
CON	487,006	54,860	397	2,700	1,142.92	100	1,458.90
HD	491,206	51,846	254	2,226	1,036.92	100	

Abbreviations: CON = control ($n = 48$); HD = high stocking density ($n = 50$).

¹ Data were pre-processed using DADA2 plugin in QIIME 2.



Supplementary Fig. S2. Alpha-rarefaction curves for observed features in nasal (A) and fecal (B) samples of finishing pigs. Sequencing depths were at 2,208 and 254, respectively. Abbreviations: CON = control ($n = 48$); HD = high stocking density ($n = 50$).

Supplementary Table S3. Effect of stocking density on nasal and fecal microbiota alpha-diversity in finishing pigs.

Alpha-diversity indices	Mean ¹		<i>P</i> -value ²
	CON	HD	
Nasal			
Shannon Entropy	4.56 ± 0.081	4.34 ± 0.061	0.009*
Simpson	0.92 ± 0.007	0.92 ± 0.004	0.060
Fecal			
Shannon Entropy	2.05 ± 0.083	1.93 ± 0.097	0.325
Simpson	0.64 ± 0.021	0.61 ± 0.025	0.611

Abbreviations: CON = control (n = 48); HD = high stocking density (n = 50).

¹ Mean ± SEM (standard error of mean)

² The *P*-values were determined using Mann-Whitney U test (*P* < 0.05).

* Statistically significant *P*-value.

Supplementary Table S4. Relative abundance (%) at phylum, genus and species levels in nasal samples of finishing pigs.

Taxa	Mean ¹		P-value ²
	CON	HD	
Phylum			
Proteobacteria	50.77 ± 2.058	50.12 ± 1.602	0.642
Firmicutes	30.66 ± 1.940	29.56 ± 2.135	0.248
Bacteroidota	12.89 ± 1.379	10.26 ± 1.266	0.148
Actinobacteriota	5.50 ± 0.758	9.96 ± 1.112	0.005*
Patescibacteria	0.09 ± 0.024	0.09 ± 0.030	0.604
Fusobacteriota	0.09 ± 0.082	0.005 ± 0.005	0.528
Genus			
<i>Moraxella</i>	36.44 ± 2.333	38.14 ± 1.947	0.534
<i>Streptococcus</i>	12.57 ± 0.840	11.40 ± 1.208	0.073
<i>Rothia</i>	5.50 ± 0.758	9.94 ± 1.109	0.005*
<i>Bergeyella</i>	6.68 ± 1.127	6.58 ± 1.099	0.823
<i>Actinobacillus</i>	9.09 ± 1.413	4.26 ± 0.980	0.001*
<i>Companilactobacillus</i>	4.37 ± 1.243	6.20 ± 1.391	0.332
<i>Filobacterium</i>	5.16 ± 0.949	2.33 ± 0.693	0.001*
Carnobacteriaceae unassigned	3.61 ± 0.621	3.40 ± 0.597	0.703
Neisseriaceae unassigned	2.69 ± 0.417	2.71 ± 0.485	0.974
<i>Mannheimia</i>	1.98 ± 0.547	3.29 ± 1.003	0.208
<i>Levilactobacillus</i>	1.53 ± 0.487	2.04 ± 0.569	0.709
<i>Lactobacillus</i>	1.22 ± 0.234	1.89 ± 0.862	0.057
<i>Clostridium sensu stricto</i> 1	1.63 ± 0.276	1.23 ± 0.270	0.267
<i>Chryseobacterium</i>	0.69 ± 0.201	1.33 ± 0.302	0.062
<i>Terrisporobacter</i>	0.82 ± 0.141	0.65 ± 0.132	0.351
<i>Limosilactobacillus</i>	0.69 ± 0.183	0.67 ± 0.213	0.311
<i>Lactiplantibacillus</i>	0.80 ± 0.196	0.55 ± 0.154	0.222
<i>Mycoplasma</i>	1.21 ± 0.325	0.17 ± 0.071	<0.001*
<i>Weissella</i>	0.88 ± 0.448	0.15 ± 0.080	0.326
<i>Neisseria</i>	0.43 ± 0.169	0.43 ± 0.179	0.916
<i>Acinetobacter</i>	0.13 ± 0.103	0.62 ± 0.543	0.689
<i>Romboutsia</i>	0.37 ± 0.092	0.21 ± 0.098	0.067
<i>Psychrobacter</i>	0	0.55 ± 0.312	0.026*
<i>Globicatella</i>	0.13 ± 0.032	0.36 ± 0.087	0.053
Other genera	1.38 ± 0.531	0.90 ± 0.221	0.513
Species			
<i>Moraxella</i> sp.	16.02 ± 1.757	17.96 ± 1.620	0.240
<i>Moraxella</i> unassigned	11.51 ± 1.646	12.09 ± 1.083	0.288
<i>Streptococcus suis</i>	9.60 ± 0.871	8.64 ± 0.985	0.137
<i>Rothia nasimurium</i>	5.48 ± 0.759	9.94 ± 1.109	0.005*

<i>Moraxella pluranimalium</i>	4.89 ± 0.911	6.77 ± 1.138	0.135
<i>Glaesserella parasuis</i>	6.85 ± 1.083	2.91 ± 0.564	0.002*
<i>Lactobacillus paralimentarius</i>	3.53 ± 1.025	5.47 ± 1.287	0.356
<i>Filobacterium</i> uncultured	5.16 ± 0.949	2.33 ± 0.693	0.001*
<i>Carnobacteriaceae</i> unassigned	3.61 ± 0.621	3.40 ± 0.597	0.703
<i>Bergeyella zoohelcum</i>	4.25 ± 1.130	2.49 ± 0.791	0.138
<i>Bergeyella porcorum</i>	2.43 ± 0.461	4.08 ± 0.925	0.123
<i>Neisseriaceae</i> unassigned	2.69 ± 0.417	2.71 ± 0.485	0.974
<i>Mannheimia</i> sp.	1.98 ± 0.548	3.29 ± 1.003	0.180
<i>Moraxella boevrei</i>	4.02 ± 1.058	1.32 ± 0.362	0.007*
<i>Actinobacillus</i> unassigned	2.22 ± 0.599	1.35 ± 0.481	0.010*
<i>Streptococcus pluranimalium</i>	2.28 ± 0.520	1.06 ± 0.279	0.024*
<i>Lactobacillus amylovorus</i>	1.06 ± 0.192	1.86 ± 0.862	0.063
<i>Clostridium sensu stricto</i> 1 uncultured	1.46 ± 0.237	1.15 ± 0.253	0.220
<i>Streptococcus acidominimus</i>	0.67 ± 0.243	1.67 ± 0.816	0.427
<i>Lactobacillus namurensis</i>	0.87 ± 0.400	1.35 ± 0.471	0.302
<i>Chryseobacterium</i> unassigned	0.69 ± 0.201	1.33 ± 0.302	0.062
<i>Terrisporobacter</i> uncultured	0.82 ± 0.141	0.65 ± 0.200	0.351
<i>Lactiplantibacillus</i> unassigned	0.79 ± 0.185	0.55 ± 0.154	0.222
<i>Companilactobacillus</i> unassigned	0.64 ± 0.255	0.65 ± 0.200	0.571
<i>Mycoplasma hyorhinis</i>	0.95 ± 0.315	0.15 ± 0.071	0.001*
<i>Psychrobacter faecalis</i>	0	0.51 ± 0.285	0.026*
<i>Mesomycoplasma flocculare</i>	0.26 ± 0.118	0.02 ± 0.013	0.017*
<i>Lactobacillus songhuajiangensis</i>	0.11 ± 0.048	0	0.001*
<i>Lactobacillus heilongjiangensis</i>	0.02 ± 0.012	0	0.039*
<i>Bacteroides xylanisolvens</i>	0.05 ± 0.019	0	0.001*
Other species	5.10 ± 0.898	4.30 ± 0.824	0.752

Abbreviations: CON = control (n = 48); HD = high stocking density (n = 50).

¹ Mean ± SEM (standard error of mean)

² The *P*-values were determined using Mann-Whitney U test (*P* < 0.05).

* Statistically significant *P*-value.

Supplementary Table S5. Relative abundance (%) at phylum, genus and species levels in fecal samples of finishing pigs.

Taxa	Mean ¹		P-value ²
	CON	HD	
Phylum			
Firmicutes	94.66 ± 1.420	94.87 ± 1.328	0.905
Bacteroidota	3.94 ± 1.229	3.86 ± 1.085	0.936
Proteobacteria	0.77 ± 0.632	0.51 ± 0.401	0.467
Actinobacteriota	0.14 ± 0.094	0.32 ± 0.169	0.708
Desulfobacterota	0.17 ± 0.174	0.25 ± 0.231	0.592
Planctomycetota	0.26 ± 0.139	0.14 ± 0.067	0.722
Spirochaetota	0.06 ± 0.042	0.02 ± 0.010	0.730
Verrucomicrobiota	0	0.02 ± 0.018	0.337
Genus			
<i>Lactobacillus</i>	45.35 ± 3.801	30.84 ± 4.336	0.019*
<i>Clostridium sensu stricto</i> 1	24.08 ± 2.773	34.33 ± 3.277	0.026*
<i>Terrisporobacter</i>	9.13 ± 1.246	15.69 ± 1.808	0.010*
<i>Limosilactobacillus</i>	6.17 ± 1.613	4.51 ± 1.044	0.628
Rikenellaceae RC9 gut group	2.64 ± 0.936	1.31 ± 0.386	0.867
<i>Romboutsia</i>	1.56 ± 0.567	2.17 ± 0.809	1.000
<i>Shuttleworthia</i>	0.68 ± 0.308	2.38 ± 1.499	0.559
<i>Dialister</i>	1.70 ± 0.572	1.05 ± 0.387	0.480
Bacteroidales p2534 18B5 gut group	0.41 ± 0.296	1.49 ± 0.812	0.208
<i>Turicibacter</i>	0.70 ± 0.198	0.76 ± 0.154	0.377
<i>Escherichia-Shigella</i>	0.77 ± 0.632	0.51 ± 0.401	0.467
[Eubacterium] coprostanoligenes group	0.93 ± 0.769	0.21 ± 0.195	0.369
<i>Sarcina</i>	1.04 ± 0.578	0	0.020*
<i>Succiniclasticum</i>	0.92 ± 0.442	0.04 ± 0.041	0.042*
<i>Prevotella</i> 7	0.27 ± 0.180	0.63 ± 0.293	0.728
<i>Streptococcus</i>	0.44 ± 0.332	0.33 ± 0.166	0.530
<i>Ruminococcus</i>	0.17 ± 0.099	0.48 ± 0.217	0.367
<i>Catenibacterium</i>	0.19 ± 0.085	0.44 ± 0.170	0.283
<i>Prevotella</i> 9	0.33 ± 0.192	0.14 ± 0.088	0.905
<i>Olsenella</i>	0.14 ± 0.094	0.29 ± 0.157	0.708
<i>Desulfovibrio</i>	0.17 ± 0.174	0.25 ± 0.231	0.592
Pirellulaceae p1088 a5 gut group	0.26 ± 0.139	0.14 ± 0.067	0.722
<i>Pseudoramibacter</i>	0.23 ± 0.137	0.11 ± 0.080	0.608
<i>Syntrophococcus</i>	0.31 ± 0.210	0	0.076
Other genera	1.41 ± 0.330	1.88 ± 0.464	0.238
Species			
<i>Lactobacillus amylovorus</i>	42.19 ± 4.023	28.21 ± 4.333	0.021*
<i>Clostridium sensu stricto</i> 1 uncultured	24.07 ± 2.773	34.12 ± 3.258	0.028*

<i>Terrisporobacter</i> uncultured	9.13 ± 1.246	15.69 ± 1.808	0.010*
<i>Lactobacillus.reuteri</i>	5.80 ± 1.559	4.51 ± 1.044	0.683
<i>Lactobacillus.johnsonii</i>	3.16 ± 1.537	2.64 ± 1.725	0.035*
Rikenellaceae RC9 gut group uncultured	2.64 ± 0.936	1.31 ± 0.386	0.867
<i>Romboutsia</i> unassigned	1.56 ± 0.567	2.17 ± 0.809	1.000
<i>Bacteroides xylanisolvens</i>	1.70 ± 0.572	1.05 ± 0.387	0.480
<i>Shuttleworthia</i> uncultured	0.50 ± 0.289	1.92 ± 1.493	0.989
Bacteroidales p2534 18B5 gut group uncultured	0.41 ± 0.296	1.49 ± 0.812	0.208
<i>Turicibacter</i> sp.	0.70 ± 0.198	0.76 ± 0.154	0.377
<i>Escherichia-Shigella</i> unassigned	0.77 ± 0.632	0.51 ± 0.401	0.467
[Eubacterium] coprostanoligenes group uncultured	0.90 ± 0.769	0.20 ± 0.195	0.300
<i>Sarcina</i> unassigned	1.04 ± 0.578	0	0.020*
<i>Succiniclasticum</i> uncultured	0.92 ± 0.442	0.04 ± 0.041	0.042*
<i>Prevotella 7</i> uncultured rumen	0.27 ± 0.180	0.63 ± 0.293	0.728
<i>Catenibacterium mitsuokai</i>	0.19 ± 0.085	0.44 ± 0.170	0.283
<i>Desulfovibrio</i> metagenome	0.17 ± 0.174	0.25 ± 0.231	0.592
Pirellulaceae p1088 a5 gut group uncultured	0.26 ± 0.139	0.14 ± 0.67	0.722
<i>Shuttleworthia</i> uncultured rumen	0.16 ± 0.124	0.22 ± 0.109	0.436
<i>Lactobacillus mucosae</i>	0.37 ± 0.367	0	0.317
<i>Ruminococcus</i> unassigned	0.15 ± 0.098	0.19 ± 0.135	0.768
<i>Streptococcus alactolyticus</i>	0.01 ± 0.012	0.32 ± 0.166	0.177
<i>Prevotella 9</i> unassigned	0.25 ± 0.177	0.08 ± 0.064	0.942
Other species	2.66 ± 0.678	3.09 ± 0.688	0.340

Abbreviations: CON = control (n = 48); HD = high stocking density (n = 50).

¹ Mean ± SEM (standard error of mean)

² The *P*-values were determined using Mann-Whitney U test (*P* < 0.05).

* Statistically significant *P*-value.

Supplementary Table S6. Effect of stocking density on pig gut microbiota-derived lactate and SCFAs concentrations.

Metabolites	Mean ¹		<i>P</i> -value ²
	CON	HD	
Lactate	16.93 ± 3.159	12.37 ± 3.538	0.005*
Acetate	199.80 ± 8.464	189.88 ± 5.658	0.548
Propionate	125.25 ± 5.616	115.89 ± 4.545	0.207
Butyrate	60.82 ± 3.333	57.06 ± 2.858	0.596

Abbreviations: CON = control (n = 48); HD = high stocking density (n = 50).

¹ Mean ± SEM (standard error of mean)

² The *P*-values were determined using Mann-Whitney U test (*P* < 0.05).

* Statistically significant *P*-value.