Title: Removal of protozoa, opportunistic pathogens with virulence factors in swine manure using anaerobic digestion: Full-scale investigation and lab-scale optimization

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Abastact: Swine manure serves as a significant reservoir of zoonotic protozoa and opportunistic pathogens, posing environmental and public health risks when inadequately treated. In this study, multiple molecular approaches, including quantitative PCR, nested PCR with *gp60*-based phylogenetic analysis, virulence factor profiling, and metagenome-assembled genome (MAG) reconstruction, were employed to investigate the abundance, diversity, and treatment responses of *Cryptosporidium*, *Giardia*, twelve opportunistic pathogens, and associated virulence factors (VFs) in swine manure. Three full-scale anaerobic digestion (AD) systems were investigated, and thermophilic and hyperthermophilic pretreatments were applied to lab-scale AD systems to evaluate the efficiency of biological risk control. *Cryptosporidium parvum* was identified as the dominant species, with subtype IIaA17G4R1 and related zoonotic subtypes detected in both lab-scale and full-scale samples. Phylogenetic clustering of swine-derived sequences with human and cattle isolates indicates a potential risk of zoonotic transmission through manure-associated environmental contamination. In lab-scale AD, a significant reduction in *Cryptosporidium*, particularly under hyperthermophilic conditions, was observed, while *Giardia* was undetectable in both influent and effluent samples. In full-scale systems, persistence of *Escherichia coli*, *Clostridium perfringens*, *Enterococcus*, *Salmonella*, and multiple VFs was confirmed in the effluents. The hyperthermophilic-mesophilic (70 °C-37 °C) lab-scale treatments achieved a substantial reduction in overall pathogen abundance from 3.40×10^8 to 1.21×10^8 copies/g dry weight and in virulence gene loads from 5.24 to 2.35 copies/cell ($P < 0.001$), along with the significant removal of pathogenic MAGs such as *Enterococcus*, *Escherichia*, *Pseudomonas*, and *Streptococcus*. These findings demonstrate the effectiveness of AD for reducing microbial risks and underscore the potential of thermophilic phase digestion as a scalable, biologically effective method for reducing microbial risks associated with livestock manure reuse.

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