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RESEARCH ARTICLE

Short Chain Fatty Acids and Bacterial Taxa Associated with Reduced *Salmonella enterica* serovar I 4,[5],12:i:- Shedding in Swine Fed a Diet Supplemented with Resistant Potato Starch

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ABSTRACT *Salmonella enterica* serovar I 4,[5],12:i:- is a foodborne pathogen of concern because many isolates are multidrug-resistant (resistant to ≥3 antimicrobial classes) and metal tolerant. In this study, three in-feed additives were individually tested for their ability to reduce *Salmonella* I 4,[5],12:i:- shedding in swine: resistant potato starch (RPS), high amylose corn starch, and a fatty acid blend, compared with a standard control diet over 21 days. Only RPS-fed pigs exhibited a reduction in *Salmonella* fecal shedding, different bacterial community compositions, and different cecal short chain fatty acid (SCFA) profiles relative to control animals. Within the RPS treatment group, pigs shedding the least *Salmonella* tended to have greater cecal concentrations of butyrate, valerate, caproate, and succinate. Additionally, among RPS-fed pigs, several bacterial taxa (*Prevotella_7*, *Olsenella*, and *Bifidobacterium,* and others) exhibited negative relationships between their abundances of and the amount of *Salmonella* in the feces of their hosts. Many of these same taxa also had significant positive associations with cecal concentrations of butyrate, valerate, caproate, even though they are not known to produce these SCFAs. Together, these data suggest the RPS-associated reduction in *Salmonella* shedding may be dependent on the establishment of bacterial cross feeding interactions that result in the production of certain SCFAs. However, directly feeding a fatty acid mix did not replicate the effect. RPS supplementation could be an effective means to reduce multidrug-resistant (MDR) *S. enterica* serovar I 4,[5],12:i:- in swine, provided appropriate bacterial communities are present in the gut.

IMPORTANCE Prebiotics, such as resistant potato starch (RPS), are types of food that help to support beneficial bacteria and their activities in the intestines. *Salmonella enterica* serovar I 4,[5],12:i:- is a foodborne pathogen that commonly resides in the intestines of pigs without disease, but can make humans sick if unintentionally consumed. Here we show that in *Salmonella* inoculated pigs, feeding them a diet containing RPS altered the colonization and activity of certain beneficial bacteria in a way that reduced the amount of *Salmonella* in their feces. Additionally, within those fed RPS, swine with higher abundance of these types of beneficial bacteria had less *Salmonella* I 4,[5],12:i:- in their feces. This work illustrates likely synergy between the prebiotic RPS and the presence of certain gut microorganisms to reduce the amount of *Salmonella* in the feces of pigs and therefore reduce the risk that humans will become ill with MDR *Salmonella* serovar I 4,[5],12:i:-.

KEYWORDS butyrate, caproate, colonization resistance, microbiome, prebiotics, resistant starch, SCFAs, *Salmonella*, swine, valerate

Salmonella is an opportunistic pathogen that can cause, and then take advantage of, intestinal inflammation to disrupt the commensal microbiota, compromise gut barriers, and colonize host tissues (1–3). Differences in pre-existing microbial populations

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may dictate the course and severity of a *Salmonella* infection (4, 5), and successful *Salmonella* colonization of the host is linked to an inflammation-associated disturbance of the gut microbiota (1–3, 6, 7). Thus, it may be important to support beneficial microbial populations to mitigate *Salmonella* colonization in pigs.

Prebiotics are dietary compounds that can facilitate the growth and metabolic activities of beneficial microbes, usually resulting in an increase in short chain fatty acids (SCFAs) concentrations in the distal intestinal tract, a major site of *Salmonella* colonization (8, 9). Increased SCFAs can have beneficial impacts on host tissues, bolstering barrier functions, enhancing tolerance to commensal organisms (and therefore reduced inflammation), and encouraging oxidative metabolism in gut tissues (10). The combination of these effects diminishes oxygen potential at the mucosa and limits inflammation-associated reactive molecules, thereby restricting terminal electron acceptor availability required for bacterial respiration (11). In gut ecosystems with limited resources to support respiration, anaerobic fermentative microorganisms outcompete bacteria that use respiration, such as *Salmonella* (12, 13). Previous research has detailed the beneficial effects of dietary resistant potato starch (RPS) in the gut environment, and how these effects may shift ecological conditions to be less hospitable and exploitable by *Salmonella* (14, 15).

To build on our previous research (14), we investigated the ability of various in-feed additives to support beneficial microbes as well as reduce the colonization and shedding of multidrug-resistant (MDR) *Salmonella enterica* serovar I 4,[5],12:i:- (*Salmonella* I 4,[5],12:i:-) in post-nursery swine. Recently, certain lineages of *Salmonella* I 4,[5],12:i:- have become a significant concern in pig production due to their acquisition of antibiotic-, metal-, and biocide-resistance genes (16, 17). In the current study, we evaluated standard commercial swine diets containing additions of an RPS, a fatty acid-natural flavoring feed additive (FAM), or resistant corn starch (RCS) for their abilities to modify the swine intestinal microbiota, promote production of SCFAs known to benefit gut health, and limit *Salmonella* colonization and shedding.

RESULTS

Association of reduced *Salmonella* fecal shedding and tissue colonization with in-feed treatments. Following the 4-week nursery period in which pigs received diets containing either no in-feed additive (CON) or in-feed treatments of RPS, FAM, or RCS, all pigs were transported to an ABSL-2 facility, challenged with *Salmonella* I 4,[5],12:i:- (SX 240), and monitored for *Salmonella* fecal shedding and tissue colonization. The RPS group exhibited significant reductions in *Salmonella* fecal shedding at 2 and 7 dpi compared with the control animals ($P = 0.01$ and 0.04, respectively, estimated marginal means, mixed models) (Fig. 1A, B). The reductions in early shedding contributed to the significantly lower area under the log curve (AULC) (cumulative shedding) observed in the RPS group compared with the control animals ($P = 0.01$), though interindividual variation was evident (Fig. 1C, D). Neither of the other treatment groups (FAM, RCS) had significantly different shedding levels when compared with the control. These data suggest that the use of RPS could be beneficial in reducing *Salmonella* shedding levels in swine.

For tissues, the estimated colonization of the experimental groups tended to be lower than that of the control group (Fig. 2). A mixed model framework and estimated marginal means contrasts suggested the treatment groups tended to have lower levels of *Salmonella* colonization compared with the control across most tissues. Significant differences in colonization levels were detected between the FAM and control group in the cecal contents ($P = 0.002$), while all groups had significantly lower colonization levels in the tonsils compared to the control ($P < 0.001$).

Alteration of microbial communities in RPS, FAM, and RCS fed pigs. Many differences in the bacterial communities were detected between the treatments and control group, but the RPS-associated communities exhibited the most differences relative to the controls (Fig. 3). We observed a significant reduction in alpha diversity in the RPS group relative to the control at days 0, 2, 14, and 21, while the FAM group exhibited reduced alpha diversity at day 0 only (Fig. 3A). We detected significant differences in the composition of fecal bacterial communities in all experimental groups compared with the control

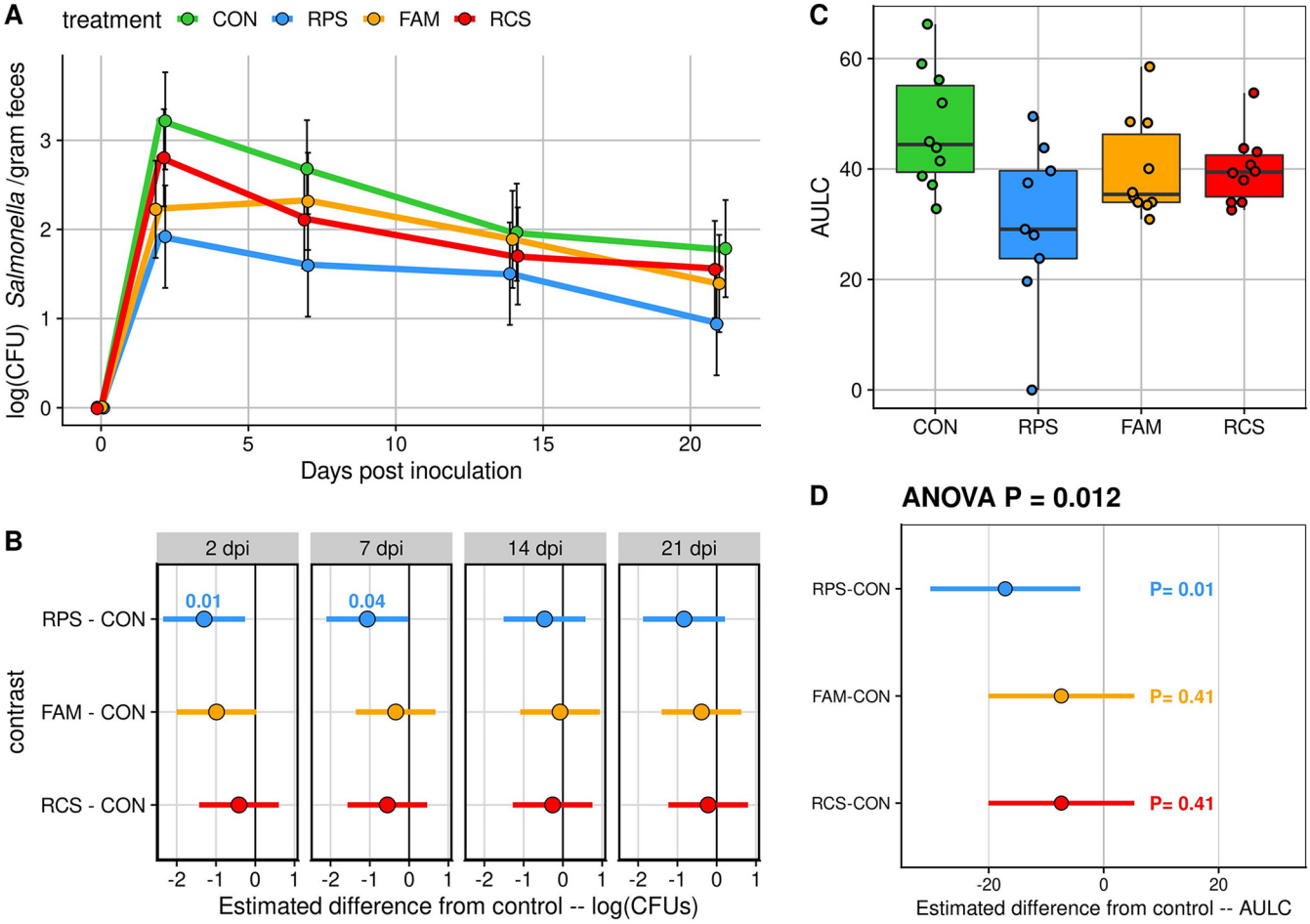

FIG 1 *Salmonella* fecal shedding throughout the challenge experiment. (A) Estimates of mean fecal shedding for each treatment group over time. Error bars represent 95% confidence intervals for the estimate of daily treatment means. (B) 95% confidence intervals for the estimated difference in shedding between each treatment and the control group. *P* values are shown when less than 0.05. (C) Estimates of cumulative fecal shedding (area under the log curve, AULC) for the 21-day experiment. (D) 95% confidence intervals for the estimated difference between the cumulative shedding for each treatment group compared with the control group. Negative estimates and confidence intervals indicate reduced shedding relative to the control group. CON, control; RPS, resistant potato starch; FAM, fatty acid mix; RCS, resistant corn starch.

group prior to inoculation (pairwise PERMANOVA FDR corrected *P* values $<$ 0.05); however, upon *Salmonella* challenge, the microbial communities of the RCS and FAM dietary treatment groups became indistinguishable from control associated communities, excluding a small difference in the RCS group at 7 dpi (Fig. 2B, Table S2, Fig. S1). In contrast, the RPS group maintained significantly different microbial communities from the control group at all time points pre- and post-*Salmonella* challenge.

***Salmonella* inoculation induced changes in gut communities.** We investigated within group changes over time by comparing the pre-inoculation communities (D0), to each of the time points, within each of the treatments via pairwise PERMANOVA tests. This analysis indicated that, at 7 dpi the bacterial communities of all groups differed from their D0 pre-inoculation status (Fig. S2, Table S3, Fig. S3). Additionally, all groups except the CON group experienced changes in their bacterial communities that persisted to 21 dpi. Although the RPS group maintained consistently different microbial communities compared to the control animals, the *Salmonella* inoculation appears to have impacted microbial community structure across all groups.

In the RPS group, a substantial variation in *Salmonella* shedding levels was observed between the pigs. When the RPS-fed animals were divided into high and low shedders (based on AULC), only the pigs that shed low levels of *Salmonella* had significantly different microbial communities from the control over the course of the study, including

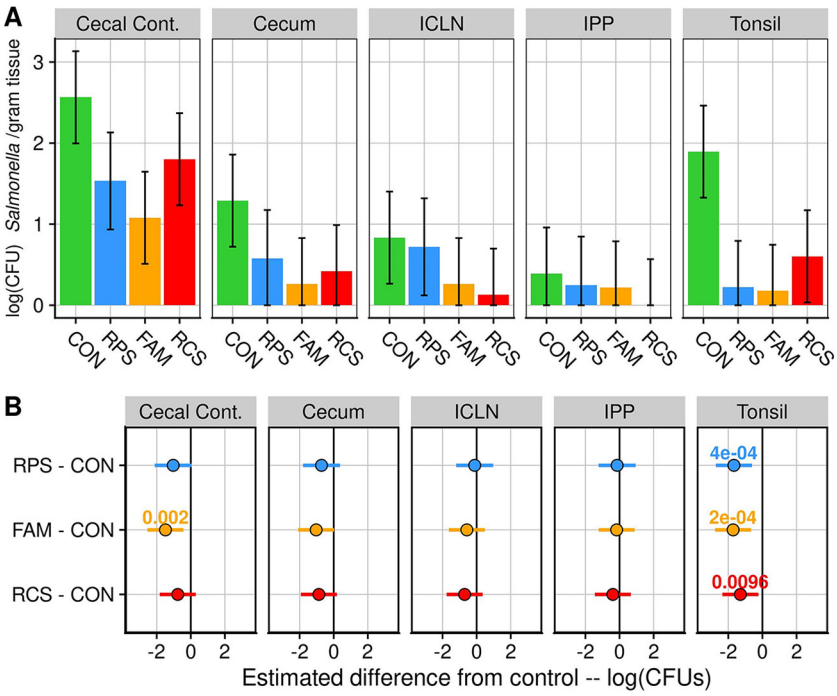

FIG 2 Estimates of *Salmonella* tissue colonization at 21 dpi. (A) Estimated mean colonization levels for each treatment group. Error bars represent 95% confidence intervals for the estimated colonization of each group. (B) 95% confidence intervals for the estimated difference between the control group and each treatment. *P* values are shown when less than 0.05. Cecal_cont, cecal contents; Cecum, cecal tissue; ICLN, ileocecal lymph node; IPP, ileal Peyer's patch region; Tonsil, palatine tonsil; CON, control; RPS, resistant potato starch; FAM, fatty acid mix; RCS, resistant corn starch.

before *Salmonella* inoculation (Fig. S4, Table S4, Fig. S5). In other words, the microbial communities in the RPS-fed animals with the lowest level of *Salmonella* shedding differed significantly from the microbial communities in the control pigs, while the microbial communities in the highest shedding animals in the RPS group were indistinguishable from those in the control group including prior to *Salmonella* inoculation (D0). This suggests that the pigs with bacterial communities that were detectably altered by RPS intake were those that shed less *Salmonella*, while those pigs with minimal change to their gut communities with RPS intake (i.e., more similar to the CON group) shed more *Salmonella*. The interindividual variation in response to RPS intake is explored further in subsequent sections.

Although significantly differentially abundant OTUs were detected between each experimental group and the control group, a greater number of significantly different OTUs were identified between the RPS and control group (Fig. 3C). Within the RPS group, OTUs from the orders *Clostridiales*, *Bacteroidales*, *Selenomonadales*, *Coriobacteriales*, *Erysipelotrichales*, *Bifidobacteriales*, *Aeromonadales*, and "unclassified *Gamma-Protoebacteria*," were enriched relative to the control group. In the FAM group, OTUs enriched relative to the control were mainly from the orders *Clostridiales*, *Bacteroidales*, and *Selenomonadales*, while OTUs enriched relative to the controls in the RCS group were mostly from the orders *Clostridiales*, *Bacteroidales*, and *Erysipelotrichales*.

Collectively, these results suggest that the RPS diet had the most striking impacts on gut microbial communities, while the impact of the other in-feed treatments differed only slightly from the control diet. OTUs enriched in the RPS-fed animals were taxonomically diverse, but were from expected gut-associated taxonomic groups. Together, these data provide further evidence that gut microbial community composition and functions may modulate the ability of *Salmonella* to colonize gut ecosystems and non-antibiotic dietary additives in combination with existing microbial communities can impact pathogen shedding phenotypes.

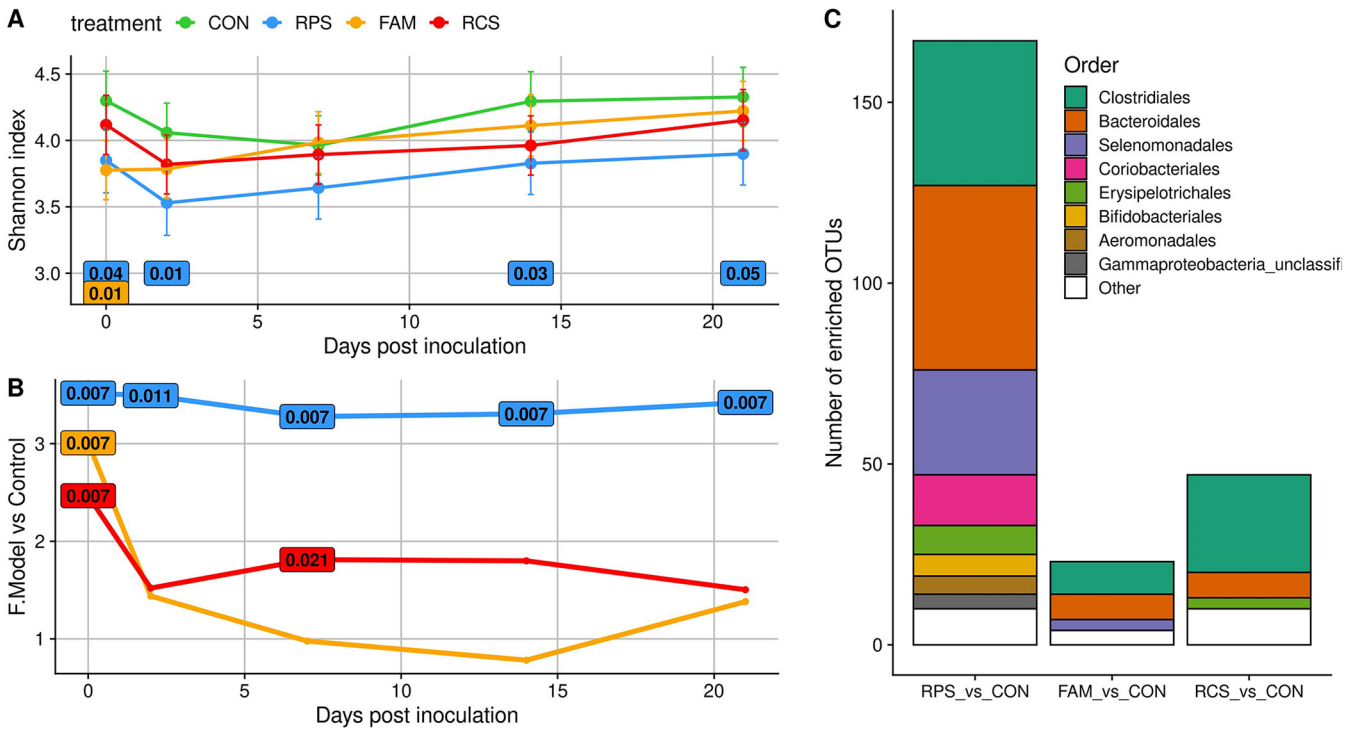

FIG 3 Differences in the fecal microbial communities for each treatment group over the course of the study. (A) Shannon diversity index values for each treatment group over time, error bars indicate 95% confidence intervals for the estimate of daily means. Higher Shannon index values indicate a more diverse fecal microbiota. (B) PERMANOVA F statistics for each group compared with the control at each time point. FDR corrected *P* values are shown when less than 0.05. A greater PERMANOVA F versus control (Y axis) indicates a greater difference in bacterial community composition relative to the control group. (C) The number of significantly enriched OTUs in each treatment group compared with the controls across all tissues and time points. Note that the *y* axis does not represent the abundance of these OTUs, but only the number of significantly differentially abundant OTUs relative to the control group. CON, control; RPS, resistant potato starch; FAM, fatty acid mix; RCS, resistant corn starch.

OTUs enriched in the RPS group belong to health-associated taxa and are associated with resistant starch intake. Many of the OTUs enriched in the RPS group relative to the control group were expected members of the swine gut microbiota and previously associated with gut health and RPS administration (14, 15, 18–24). OTUs belonging to genera *Megasphaera*, *Acidaminococcus*, *Dialister*, *Syntrophococcus*, *Olsenella*, *Bifidobacteria*, and *Prevotella_7* were enriched in the RPS group across multiple time points and tissues, with sporadic enrichments of other OTUs (Fig. 4A and B). Some OTUs enriched in the RPS group were relatively lowly abundant, including some in the orders *Clostridia*, *Gammaproteobacteria*, *Actinobacteria*, *Spirochaetia*, and *Methanobacteria*. Other OTUs enriched in the RPS group belonged to more highly abundant taxa such as those in the *Negativicutes* and *Bacteroidia* orders. In total, the microbes enriched in the RPS group relative to the control group accounted for almost half of the aggregate community (Fig. 4C). Thus, RPS intake impacted a large portion of the total community including both lowly abundant and highly abundant OTUs.

RPS intake resulted in higher cecal concentrations of butyrate, valerate, and caproate. Concomitant to the differences in microbial communities observed between the RPS-fed animals and the control animals, the cecal SCFA profiles of the RPS-fed pigs differed substantially from the control animals. Significantly higher concentrations of butyrate ($P = 0.002$), valerate ($P = 0.008$), and caproate ($P = 0.042$) were measured in the cecal contents of RPS-fed animals relative to the control on dpi 21 (Fig. 5A and B). No significant differences were detected in the other treatment groups compared to controls. When comparing cumulative fecal shedding (AULC) to the cecal concentrations of SCFAs across all treatment groups, pigs that shed the least *Salmonella* tended to have greater concentrations of butyrate, valerate and caproate in their cecal contents at 21 dpi (Fig. S6). This global correlation structure is at least partially due to the increased concentrations of butyrate, valerate, and caproate and the reduced AULC in

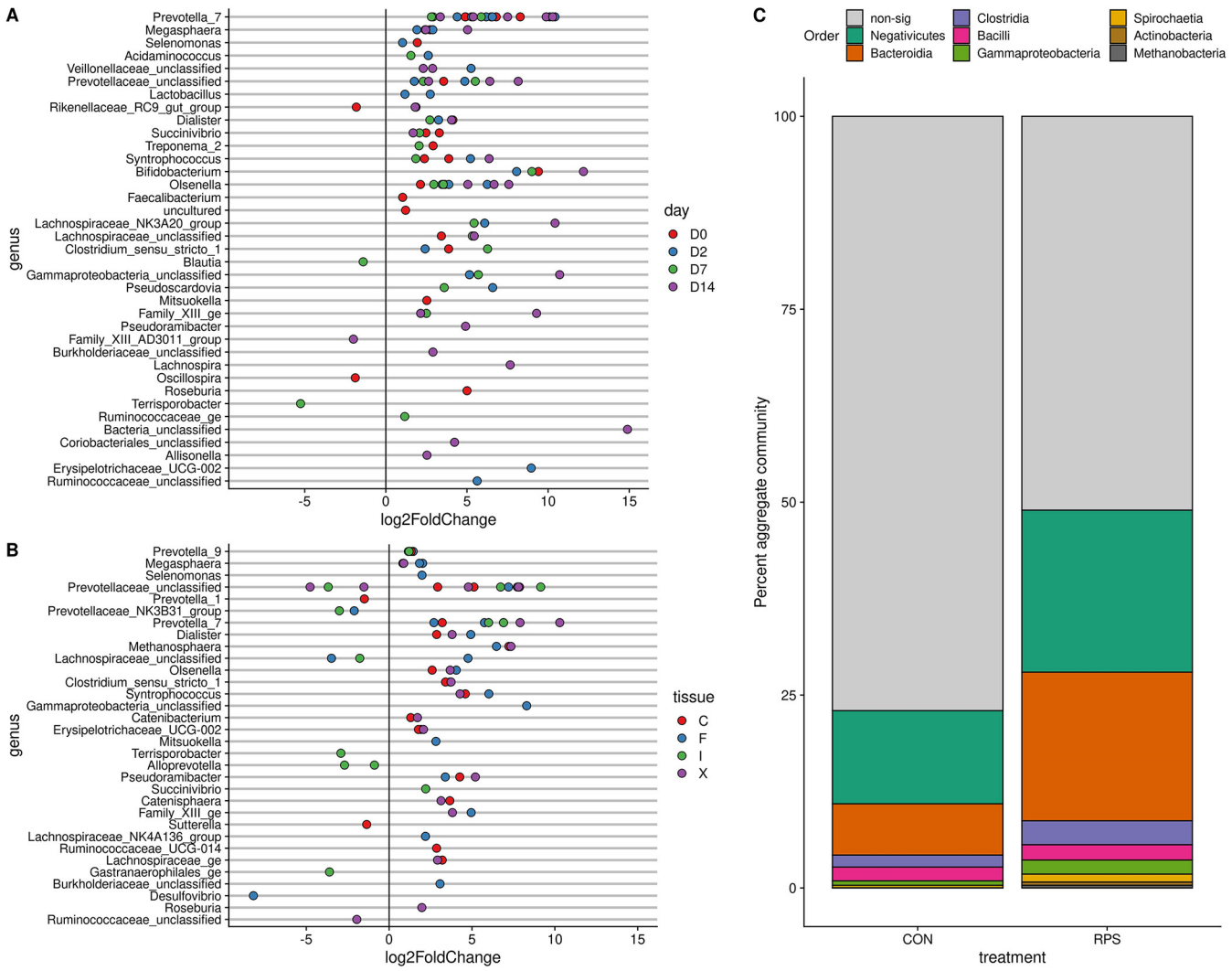

FIG 4 OTUs with significantly different abundances between the RPS and control groups throughout the study. (A) Significantly differentially abundant OTUs in fecal communities from 0 to 14 dpi. (B) Significantly differentially abundant OTUs in tissues at 21 dpi. C, cecal contents; F, feces; I, ileal mucosa; X, cecal mucosa. In both panels A and B, a positive log2FoldChange indicates the OTU is more abundant in the RPS treatment group, and the more abundant genera are positioned at the top of the *y* axis and the least abundant genera are at the bottom. (C) The percent of the aggregate community represented by OTUs enriched in the RPS group. The aggregate community represents average OTU abundances across all fecal samples and tissues. CON, control; RPS, resistant potato starch.

the RPS group. When the correlations between these SCFAs and AULC were calculated in the RPS group alone, similar patterns emerged. Considering only the RPS group, pigs that shed the least *Salmonella* were those that had the highest concentrations of these SCFAs (Fig. 5C). The trend was strongest for valerate ($R = -0.70$, $P = 0.04$) and caproate ($R = -0.74$, $P = 0.02$), while butyrate ($R = -0.45$, $P = 0.22$) was not as strongly associated with reduced cumulative shedding in the RPS group. This recalculation of correlations in the RPS group in isolation also revealed a near significant correlation between increased cecal succinate and reduced AULC ($R = -0.65$ $P = 0.06$). These results suggest that increased SCFAs in the RPS group was associated with the reduction in *Salmonella* fecal shedding, though a direct cause and effect cannot be established.

Association of specific OTUs with reduced levels of *Salmonella* and elevated concentrations of SCFAs. We sought to determine if specific bacterial OTUs were associated with SCFAs of interest and/or measurements of *Salmonella* fecal shedding. Using generalized linear models as implemented in DESeq2, we calculated regression coefficients modeling the relationship between OTU abundances and SCFA concentrations in the cecal contents, as well as the associations between OTU abundances and

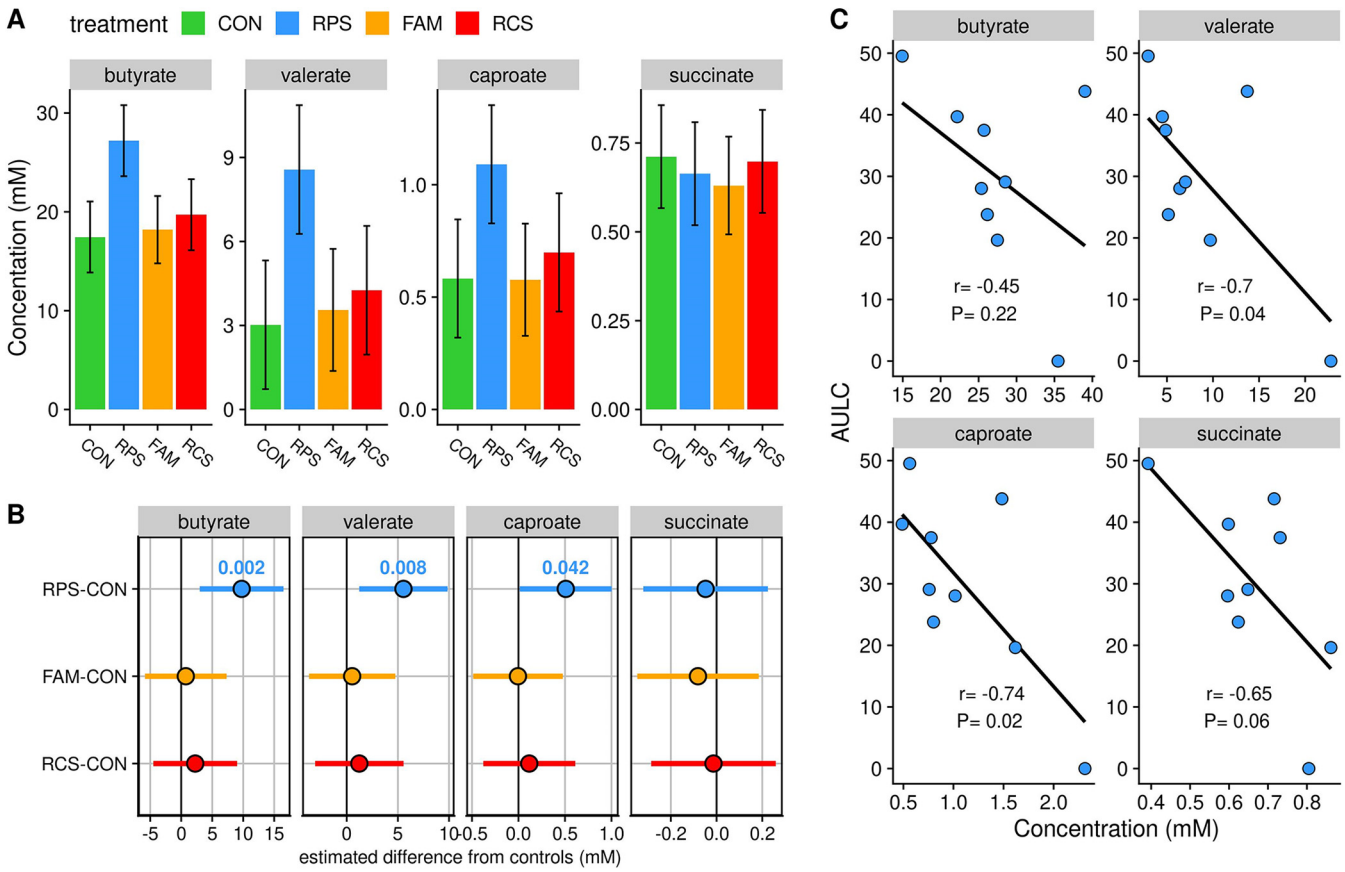

FIG 5 Cecal SCFA concentrations at 21 dpi. (A) Mean group concentrations, error bars show 95% confidence intervals for estimates of group means. (B) 95% confidence intervals for the estimated difference between each treatment group and the control group. *P* values are shown when less than 0.05. (C) Correlations between cecal SCFA concentrations and cumulative *Salmonella* shedding (AULC, area under the log curve) in the RPS-fed pigs. The correlation coefficients (r) and *P* values (P) are shown for each SCFA. CON, control; RPS, resistant potato starch; FAM, fatty acid mix; RCS, resistant corn starch.

measures of *Salmonella* shedding (log_{10} CFU/g feces, and AULC) across all samples. The regression coefficients were calculated taking treatment into account, that is, correcting for differences in SCFAs or *Salmonella* shedding across treatments. A network displaying significant associations between OTU abundances and covariates of interest was constructed (Fig. S7), with nodes representing features of interest (OTUs, SCFAs, and *Salmonella* shedding), and edges representing significant regression coefficients. The network was broadly split into two sub-networks: nodes associated with measures of increased *Salmonella* fecal shedding and decreased SCFA concentrations; and nodes associated with decreased *Salmonella* shedding and increased SCFA concentrations. Considering the relationships between OTU abundances and fecal shedding or SCFA concentrations across all treatments, many of the OTUs associated with measures of reduced *Salmonella* shedding were also associated with increased cecal concentrations of butyrate, valerate, and caproate. Additionally, many of the same OTUs were significantly enriched in the RPS-fed animals relative to control animals. A few OTUs held central positions in the network and linked both measures of reduced *Salmonella* fecal shedding and increased concentrations of SCFAs. These notable OTUs belonged to the genera *Megasphaera*, *Dialister*, *Prevotella_7*, *Olsenella*, *Selenomonas*, *Pseudoramibacter*, and *Lachnospiracaea_ge*. A *Sutterella* OTU was significantly associated with both increased *Salmonella* fecal shedding and reduced butyrate. Overall, the network represents important relationships between OTUs, SCFAs and reduced *Salmonella* shedding across all groups.

Because only the RPS treatment group exhibited reduced *Salmonella* fecal shedding, another network was constructed using data only from the RPS-fed pigs to

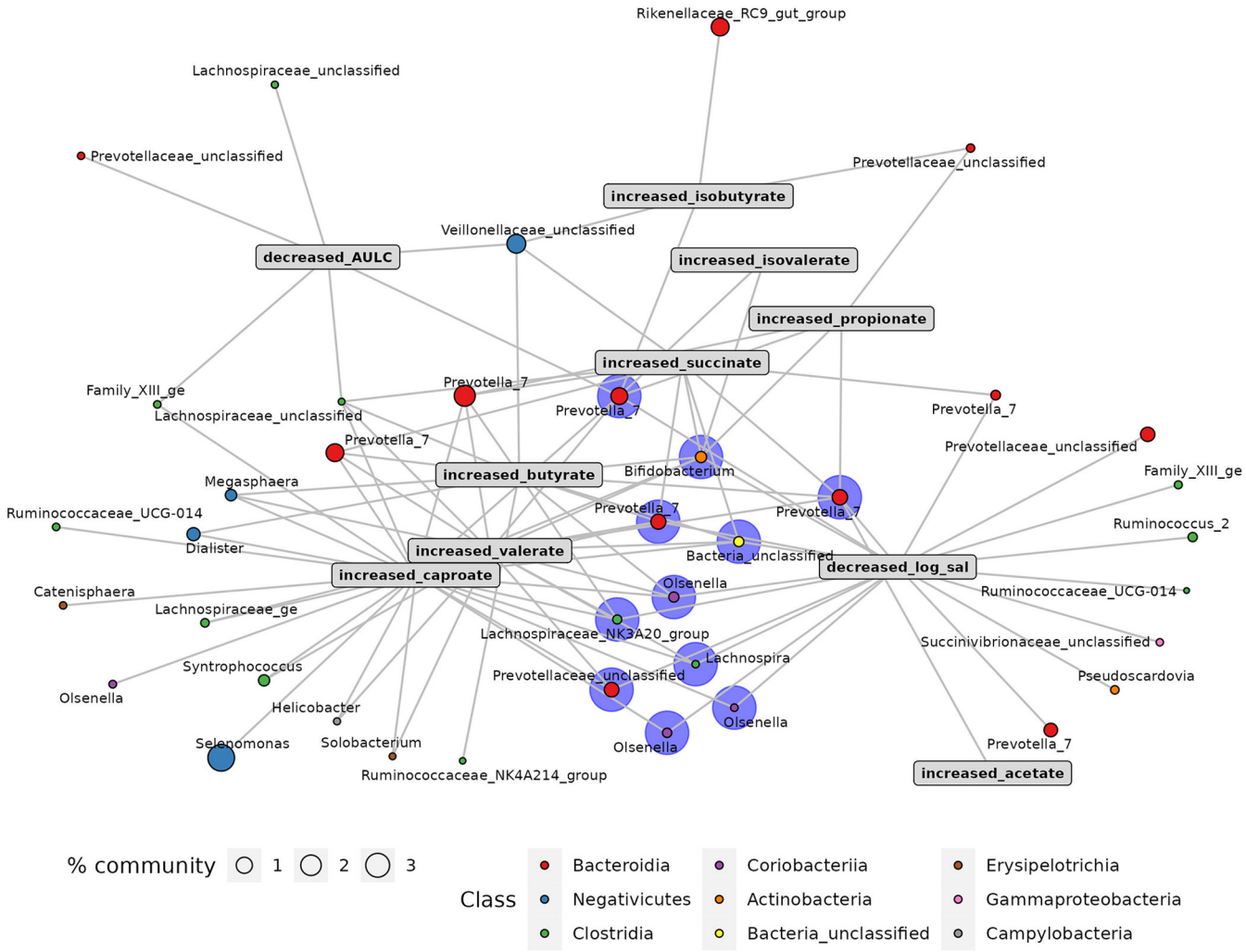

FIG 6 A network of associations between SCFAs, measurements of *Salmonella* fecal shedding, and OTU abundances using data from RPS-fed pigs only. Associations with *Salmonella* shedding were calculated from each sample with matched *Salmonella* data, SCFA associations were calculated from cecal content samples. Nodes represent OTUs and covariates and edges represent significant associations (regression coefficients). Associations were calculated with regression models implemented by DESeq2. *P* values for calculated coefficients were corrected by the FDR method considering all tests performed. Coefficients with FDR corrected *P* values > 0.05 and $\log_2$ fold changes less than 0.5 were removed prior to network construction. OTU nodes are sized according to their aggregate abundance across all samples (% community). Only one neighborhood of this network is shown for readability, the full network is available in supplement (Fig. S8). OTUs that link increased concentrations of butyrate, valerate, and caproate with reduced *Salmonella* shedding are shown with a blue background. These OTUs may be important in producing the low shedding, high SCFA phenotype seen in some RPS-fed animals.

determine if the relationships between OTUs, SCFAs, and *Salmonella* shedding differed in the context of a RPS diet. To enable easier viewing of important features in the network, only the nodes and edges in the community associated with reduced *Salmonella* fecal shedding and increased SCFAs are shown (Fig. 6). The full network is available in the supplement (Fig. S8). The RPS-only network revealed many of the same associations evident in the network of all treatment groups; however, some differences were apparent. Several OTUs held central positions in the RPS-only network, meaning they linked different covariates of interest. Nodes with the highest degree (number of edges) are likely to be important members, such as *Prevotella_7*, *Bifidobacterium*, *Olsenella*, and *Lachnospiraceae* OTUs. Associations between central OTUs and succinate concentrations emerged when considering the RPS-only data. Overall, the RPS-only network analysis suggests the RPS-fed animals with greater abundances of the aforementioned OTUs had lower levels of *Salmonella* fecal shedding and higher cecal concentrations of butyrate, valerate, and caproate, revealing OTUs that may play an important role in elevating SCFA levels as well as reducing *Salmonella* shedding in the context of an RPS amended diet.

DISCUSSION

The RPS response-phenotype observed in this study is in line with previous descriptions of animals fed resistant starch. A reduction in alpha diversity was observed in RPS-fed animals that parallels similar responses noted for pigs administered type 2 resistant starches in feed (23, 24). The increases in butyrate, valerate, and caproate observed in the RPS-fed animals align with our previous research of the impacts of RPS intake on the swine gut ecosystem (14). In addition, many of the OTUs associated with the phenotype of interest in this study, belong to the same taxonomic groups enriched in the RPS-fed pigs in our previous study. For example, OTUs belonging to the genera *Megasphaera*, *Prevotella_7*, *Dialister*, *Acidaminococcus*, *Olsenella*, *Bifidobacterium*, and *Syntrophococcus* were identified in the RPS response-phenotype in both studies (14), despite the pigs being sourced from different herds. Other studies that administered resistant starch to various animal species detected similar increases in these taxa as well. *Olsenella* was previously associated with resistant starch intake in broilers (20) as well as swine (25). A recent clinical trial observed increases in *Bifidobacterium*, *Olsenella*, and *Prevotella* in humans fed resistant starch (19), and another study detected enrichments of *Bifidobacterium* in swine fed resistant starch (18).

While RPS-fed pigs exhibited significant shifts in bacterial communities and cecal SCFAs, feeding pigs RCS did not result in the same phenotype. In line with our results, a recent study in humans showed that dietary RPS stimulated SCFA production while a high amylose corn starch failed to do so (26). Additionally, within the RPS group of the human study, not all individuals responded uniformly, and the presence of key primary starch degraders in the microbiota was important for the increased SCFA phenotype. In humans it was suggested that bacteria belonging to the genus *Ruminococcus* were important primary degraders of RPS that developed butyrogenic cross-feeding interactions with other members of the microbiota. In addition, gut ecosystems with *Bifidobacteria* as major primary degraders did not exhibit the increased butyrate as seen in ecosystems with *Ruminococcus* as major primary degraders. The findings from our present study are somewhat different as we did not detect an association of *Ruminococcus* OTUs with the increased SCFA phenotype, but rather OTUs from the genera *Bifidobacterium*, *Prevotella_7* and *Olsenella* held central positions in our phenotype-response networks and were associated with both increased SCFAs as well as reduced *Salmonella* fecal shedding. However, in addition to differing host species between the studies, it is important to note that diversity of ecological roles exist within these genera and not all species of a genera will behave identically. Furthermore, our study was conducted in the context of a *Salmonella* disturbance which further complicates comparisons between the studies and interpretation of these interactions.

Similar to previous studies indicating that appropriate primary degraders are key in phenotypic responses to resistant starches, in our study many of the OTUs associated with increased butrayte, valerate, and caproate do not belong to taxa known to produce these compounds. Production of these three SCFAs from dietary fiber generally requires a primary degrader to perform the initial breakdown of the polymers. Secondary fermenters can then cross-feed using the released simple sugars and the metabolic outputs of primary degraders (such as acetate, lactate, and succinate) to produce the final fermentation products (27, 28). Therefore, observations of OTUs associating with SCFAs that are not typically produced by organisms represented in the OTUs may signify important primary degraders that provide simpler carbon sources to secondary fermenters that cross-feed to produce butyrate, valerate, and caproate. Our study identified OTUs belonging to the genera *Prevotella_7*, *Bifidobacterium*, and *Olsenella*, as candidate organisms that could fill this primary degrader niche. Furthermore, our results suggest that the interindividual variations observed in the RPS response-phenotype could be linked to differences in abundance of the important primary starch degraders, in line with previous work (29, 30). Specifically, the pigs with the highest concentrations of SCFAs and lowest levels of *Salmonella* fecal shedding having the most robust populations of these potential primary starch degraders.

While the SCFA butyrate is known to be a microbial metabolite of central importance, our data suggest the reduction in *Salmonella* fecal shedding in RPS-fed animals was more strongly associated with the longer SCFAs valerate and caproate. These two longer SCFAs may be indicators of conditions that favor bacteria which are fermentative specialists. It has been proposed that longer SCFAs can be produced using propionate or butyrate as substrates, with the same metabolic machinery as used in butyrate production in a reverse beta-oxidation process (31). It follows that valerate and caproate may only be produced once conditions that favor fermentative specialists are established and shorter SCFAs such as acetate, propionate, and butyrate are abundant. Additionally, while butyrate is a high priority metabolic input for intestinal epithelial cells, valerate and caproate may be higher priority substrates than butyrate for oxidation in the mucosa (32). Knowledge of the health benefits of longer SCFAs is more limited than that of butyrate, but the longer SCFAs also provide benefits to intestinal host-microbiota systems. Valerate may provide some protection from auto-immune disorders such as eczema (33), as well as a protective effect against *Clostridium difficile* infections (34). These longer, less well-characterized, SCFAs deserve further investigation into their roles in the reduction in *Salmonella* colonization and shedding.

Our phenotype association networks suggest that increased SCFA production was closely related to reduced *Salmonella* fecal shedding. Robust SCFA production plays key roles in health and barrier function in gut-associated mucosal tissues (12–14); additionally, increased concentrations of SCFAs are known to reduce the luminal pH which can have bactericidal effects on pathogens such as *Salmonella* (21). Furthermore, SCFA production by commensals and oxidation of these SCFAs by gut tissues can play a central role in driving environmental conditions in the gut toward those that favor fermentative specialists to the detriment of those microbes that prefer to use respiratory metabolisms, such as *Salmonella* (6, 12, 13). *Salmonella* relies on generating an inflammation-linked oxidative disturbance to disrupt healthy commensal ecosystems, thereby aiding in host colonization and environmental dissemination (1–3, 6, 7, 35, 36). Only the bacterial communities in RPS-fed pigs exhibited consistent differences from those in the control pigs throughout the experiment which suggests the RPS communities reacted differently to *Salmonella* inoculation compared with all other treatments.

Differences in microbial community composition within the RPS-fed pigs helps explain the variation in *Salmonella* shedding and within this group. One hypothesis is that different gut communities process RPS in distinct ways with different metabolic end products, and certain microbial functions are required for a response that most benefits the host (30, 37, 38). The activities of some communities may be more beneficial for host tissues, providing a gut environment that is more resilient to incursion by opportunistic pathogens. Previous work has identified many ecosystem services that are associated with increased pathogen resistance (11, 12, 38). Although our study design did not allow the interrogation of why certain RPS-fed pigs came to harbor communities that provided increased resistance, we observed that the RPS-fed communities that produced the most SCFAs butyrate, valerate, and caproate provided the most resistance to *Salmonella*. Not all primary degraders of resistant starch have the same capacities to share the simple sugars that result from primary starch degradation (29, 37), and the presence of other cross-feeding members can also be a major determinant of resistant starch response phenotypes (37). This study as well as previous work suggests that these primary degraders or other crossfeeding members may play a large role in determining the RPS response phenotype and therefore the *Salmonella* shedding phenotype as well.

Another possibility is that RPS-fed communities could help limit *Salmonella* numbers if they could withstand or buffer the oxidative stress *Salmonella* induces in the intestine. In line with this idea, we noted that many of the central OTUs in our RPS phenotype association network belonged to taxa able to cope with microaerobic conditions or perform some form of respiration. For example, species within both the *Bifidobacterium* and *Olsenella* genera are microaerophiles (39, 40); similarly, members

of *Prevotella_7* produce respiratory menaquinones (41, 42), suggesting they may utilize respiration when conditions allow. The ability to withstand the conditions induced by a *Salmonella* infection may have allowed the important primary starch degraders to maintain their keystone roles during the *Salmonella*-induced oxidative disturbance. The communities in higher shedding RPS-fed pigs may not have had primary degraders that could cope with the *Salmonella* disturbance. If these keystone members of the communities could not cope with oxidative stress, the foodwebs they feed would collapse which could result in reduced SCFA production and further increased oxidative conditions in which *Salmonella* can thrive. Future efforts into characterizing communities associated with an improved RPS response phenotype should include culturing efforts to better characterize the ecological niche of important members. These results highlight taxa that should be investigated for their abilities to enhance the beneficial effects of RPS and improve colonization resistance.

The presence of endogenous competitor organisms provides yet another alternative hypothesis for the within-RPS treatment variations in the *Salmonella* fecal shedding phenotype. For example, endogenous *Enterobacteriaceae* can have a major influence on the *Salmonella* colonization phenotype in mice (43). Mice already colonized by endogenous *Enterobacteriaceae* organisms prior to a *Salmonella* challenge were resistant to colonization. The hypothesized mechanism for this effect was that these pre-existing microbes had a similar respiration-based metabolism and occupied the same niche in the gut ecosystem that *Salmonella* colonizes; this nutritional competition excluded *Salmonella*. These protective microbes, such as other *Enterobacteriaceae*, competing with *Salmonella* were often of low abundance in the microbiota and generally below the limit of detection of many culture-independent approaches like 16S rRNA amplicon sequencing. Other work has suggested similar roles for *Proteobacteria* helping to maintain anaerobic gut environments in other species (44). Unfortunately, the design of our study did not allow us to speculate on the presence of such organisms in the animals shedding low levels of *Salmonella*. Future studies that seek to characterize microbial features that impact *Salmonella* colonization and shedding should consider employing culture-based or other approaches that allow the interrogation of more rare members of the microbiota.

Conclusions. This study detailed alterations in the swine gut microbiota and SCFA production through the course of a *Salmonella* challenge in the context of different in-feed additives. Only the RPS-fed group had consistent, meaningful differences when compared with the control group. The RPS-fed animals had a different *Salmonella*-induced change in their gut microbial communities, an enrichment of RPS- and health-associated microbial taxa, increased concentrations of health-associated SCFAs, and lower measurements of *Salmonella* fecal shedding relative to the control animals. Within the RPS-fed animals, interindividual variation in response phenotypes was evident. In RPS-fed pigs, those shedding the lowest levels of *Salmonella* had higher cecal concentrations of butyrate, valerate, caproate, and succinate and higher abundances of many OTUs from genera such as *Bifidobacteria*, *Olsenella*, *Prevotella_7*, and others. Collectively, the data suggest that RPS may be an effective option for limiting *Salmonella* colonization and shedding (including MDR *Salmonella* serovar I 4,[5],12:i:-) in swine, provided the appropriate bacterial communities that can utilize RPS as a substrate are present in the gut.

MATERIALS AND METHODS

Diets and experimental design. Diets were formulated to industry standards with two diet phases. Phase-1 basal diets were fed from day (d) 1 to d14 and were formulated to contain 3,400 kcal metabolizable energy/kg diet, 1.50% standardized ileal digestible lysine, 0.84% calcium, and 0.45% standardized total tract digestible phosphorus. Phase-2 diets were fed from d15 to d28 and were formulated to contain 3,400 kcal metabolizable energy/kg diet, 1.36% standardized ileal digestible lysine, 0.80% calcium, and 0.40% standardized total tract digestible phosphorus. In each phase, all other amino acids were formulated to meet minimum digestible amino acid:lysine ratios as reported in the NRC (2012). (see Table S1 for complete diet formulations). Weaned pigs, 19 to 21 days of age, obtained from a commercial swine farm were separated into treatment groups within a 96-pen, 480-pig nursery room at the Iowa State University Swine Nutrition Farm, Ames, IA. Treatment groups consisted of 10 pens of five piglets for each dietary treatment, with one pig per pen randomly selected for further inclusion in the

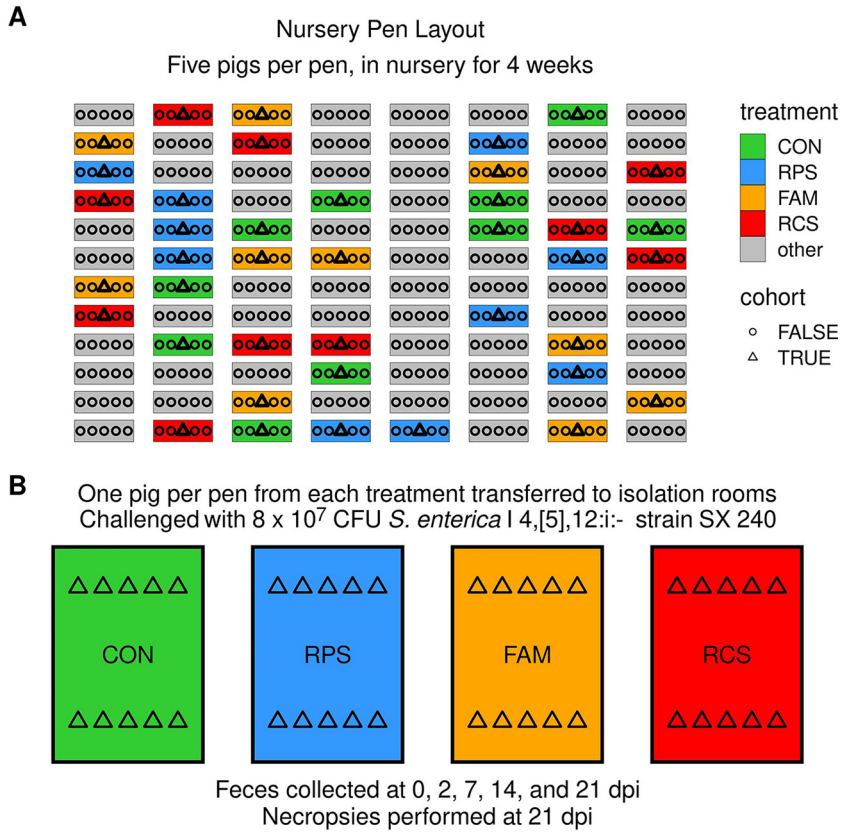

FIG 7 Overview of the experimental design. Each shape (circle or triangle) represents one pig. Triangles indicate pigs selected for the *Salmonella* challenge cohort. (A) The pen layout and cohort membership during the nursery phase of this study, from days 0 to 28 postweaning. (B) At 28 days post-weaning, cohort members were transferred to isolations rooms (one dietary treatment per room) for the *Salmonella* challenge phase of the study and inoculated with 8×10^7 CFU *S. enterica* strain SX 240 (a MDR serovar I 4,[5],12i- isolate derived from strain USDA15WA-1). Fecal samples were collected at specific time points over the 21-day experiment and necropsies performed at 21 days postinoculation (dpi). CON, control; RPS, resistant potato starch; FAM, fatty acid mix; RCS, resistant corn starch; other, animals not part of this study.

inoculation cohort, for a total of 10 piglets in each treatment group (Fig. 7). Treatments consisted of pigs fed a non-amended diet (control, CON), or a similarly formulated diet amended with either: 5% resistant potato starch (RPS; MSP Starch Products Inc., Carberry, MB, Canada), 0.30% fatty acid mix (FAM, SANACORE EN) (salts of sodium butyrate and propionate, palm fat, caprylic, capric, and lauric fatty acids, natural flavoring compounds, and a mixture of steatites and chlorite, Nutriad Inc., Hampshire, IL) or 5% resistant corn starch (RCS, HI-MAIZE 260, Ingredion Inc., Bridgewater, NJ). After pigs received their specific diets for 28 days, the selected inoculation cohort pigs were transferred to isolation rooms at the National Animal Disease Center (NADC, Ames, IA) with one dietary treatment group per isolation room (four rooms total). Pigs were inoculated via the intranasal route (a natural route of *Salmonella* exposure due to pigs rooting behavior) with 8×10^7 CFU of *Salmonella* I 4,[5],12:i:- strain SX 240 (swine passaged USDA15WA-1 strain) as previously described (17). Following inoculation, pigs continued to receive their respective phase 2 diets for the remaining 21-days. Fecal sampling intervals for microbiota and *Salmonella* qualitative and quantitative bacteriology analyses included 0-, 2-, 7-, 14-, and 21-days postinoculation (dpi). At day 0 (prior to inoculation), pigs tested fecal-negative for *Salmonella* as described previously (45). At 21 dpi, all pigs were euthanized and necropsied to obtain tissue samples (1 g) of the cecum, ileocecal lymph nodes (ICLN), Ileal Peyer's patch region (IPP), palantine tonsil, and contents of the cecum. All samples were evaluated by quantitative and qualitative bacteriology analyses for *Salmonella* I 4,[5],12:i:- as previously described (46) using XLT-4 medium (Becton, Dickinson and Company, Sparks, MD) supplemented with 50% tergitol, ampicillin (100 μg/mL), tetracycline (15 μg/mL), novobiocin (50 μg/mL), and streptomycin (50 μg/mL). *Salmonella* colonies were evaluated on BB CHROMagar *Salmonella* (Becton, Dickinson and Company) medium for mauve colonies indicative of *Salmonella*. All experimental procedures involving pigs were in compliance with the recommended principles described in the Guide for the Care and Use of Laboratory Animals by the National Research Council of the National Academies and were approved by the Institutional Animal Care and Use Committee at Iowa State University and the National Animal Disease Center.

Microbial community analysis. 16S rRNA gene amplicons of the V4 region were generated from feces, cecal contents, and cecal mucosal samples as described in (46) in accordance with the protocol

described by Kozich et al. (47). Briefly, samples were collected, immediately placed on ice, and stored at −80°C until DNA extraction with Qiagen Fecal Microbiome kits (Germantown, MD). Amplicons were sequenced on an Illumina Miseq (La Jolla, CA) using V2 reagent kits (2 × 250 bp read lengths). Operational taxonomic units (OTUs) were generated with mothur (48) and classified using the SILVA v132 taxonomy. Global singletons were removed and samples with fewer than 2,000 total reads were omitted.

Cecal SCFA measurements. One gram of cecal contents was thawed, suspended in 2 mL PBS, vortexed for 1 min, and debris was pelleted by centrifugation at 5,000 × *g* for 10 min. The resulting supernatant (1 mL) was added to heptanoic acid internal standards. Butylated-fatty acid esters were generated as previously described (49), and analyzed using an Agilent 7890 GC (Agilent, Santa Clara, CA). This assay measures the following 12 SCFAs: formate, acetate, propionate, isobutyrate, butyrate, lactate, isovalerate, valerate, caproate, oxalate, phenylacetate, succinate, and fumarate.

Statistical analysis. Statistical testing and plotting was conducted in R (50), and tidyverse (51) packages were used to assist in plotting and data wrangling. Calculation of AULC for cumulative *Salmonella* fecal shedding was performed using a trapezoidal integration method implemented in the trapz function of the pracma package (52). Differences in cecal SCFA concentrations, AULC, and tissue colonization were assessed within an ANOVA framework, 95% confidence intervals and *post hoc* tests were conducted via Tukey's honest significant differences method. The lme4 (53), lmerTest (54) and emmeans (55) packages were used to construct mixed models to assess and generate 95% confidence intervals for the estimated differences in *Salmonella* fecal shedding and alpha diversity between treatment groups and controls at each time point. The R package vegan (56) was used to assess the effect of treatment on community structure similarity through PERMANOVA tests on Bray–Curtis dissimilarities as implemented by the adonis function. NMDS ordinations were generated from these same Bray–Curtis dissimilarities. The Shannon alpha-diversity index was used to quantify alpha diversity. Communities were rarefied to 2,462 reads each before dissimilarity and alpha diversity calculations. The overall effect of treatment and time was assessed with a global test followed by individual pairwise *post hoc* tests for comparisons of interest (each treatment compared to the controls). *P*-values were corrected for multiple comparisons using the FDR method. Determination of differential abundance of OTUs relative to the control group and associations between OTU abundances and continuous covariates was accomplished using the DESeq2 package (57) using Wald tests with parametric fits and FDR-corrected *P*-values. Before model construction, OTUs with fewer than 10 counts globally were removed, and the resulting unrarefied counts were used as the input for DESeq2. Estimated coefficients were shrunk using the apeglm package (58). The package phyloseq (59) was used to assist in data wrangling. Network visualization was accomplished with the geomnet (60) package.

Data availability. All raw sequence data has been deposited in the SRA and is available under bioproject no. PRJNA638426. All scripts used for analysis and figure generation are available at https://github.com/USDA-FSEPRU/FS12b.git.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.1 MB.

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