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Resistant starch reduces large intestinal pH and promotes fecal lactobacilli and bifidobacteria in pigs

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Dietary resistant starch (RS) may have prebiotic properties but its effects on fermentation and the microbial population are inconsistent. This meta-analysis aimed to quantify the relationship between RS type 2 (RS2) and intestinal short-chain fatty acids (SCFA) and pH as well as certain key bacterial taxa for intestinal health in pigs. From the 24 included articles with sufficient information about the animal, and dietary and physiological measurements published between 2000 and 2017, individual sub-data sets for fermentation metabolites, pH, bacterial abundances and apparent total tract digestibility were built and used to parameterize prediction models on the effect of RS2, accounting for inter- and intra-study variability. In addition, the effect of pig's BW at the start of the experiment and duration of the experimental period on response variables were also evaluated using backward elimination analysis. Dietary RS levels ranged from 0% to 78.0% RS, with median and mean RS levels of 28.8% and 23.0%, respectively. Negative relationships could be established between dietary RS and pH in the large intestine ($P < 0.05$), with a stronger effect in the mid and distal colon, and feces ($R^2 = 0.64$ to 0.81; $P < 0.001$). A dietary level of 15% RS would lower the pH in the proximal, mid-, distal colon and feces by 0.2, 0.6, 0.4 and 0.6 units, respectively. Increasing RS levels, however, did not affect SCFA concentrations in the hindgut, but enhanced the molar proportion of propionate in mid-colon and reduced those of acetate in mid-colon and of butyrate in mid- and distal colon ($R^2 = 0.46$ to 0.52; $P < 0.05$). Backward elimination indicated an age-related decrease in mid-colonic propionate proportion and increase in mid- and distal colonic butyrate proportion ($P < 0.05$), thereby modulating RS2 effects. In feces, increasing RS levels promoted fecal lactobacilli ($R^2 = 0.46$; $P < 0.01$) and bifidobacteria ($R^2 = 0.57$; $P < 0.01$), whereby the slope showed the need for a minimal RS level of 10% for a 0.5 log unit-increase in their abundance. Best-fit equations further supported that a longer experimental period increased fecal lactobacilli but decreased fecal bifidobacteria ($P < 0.05$). In conclusion, dietary RS2 seems to effectively decrease digesta pH throughout the large intestine and increase lactic acid-producing bacteria in feces of pigs which may limit the growth of opportunistic pathogens in the hindgut. To achieve these physiologically relevant changes, dietary RS should surpass 10% to 15%.

Keywords: resistant starch type 2, gastrointestinal tract, meta-analysis, lactic acid-producing bacteria, short-chain fatty acids

Implications

Dietary resistant starch (RS) can negatively impact the energetic efficiency in pigs. By contrast, RS forms a type of dietary fiber that may affect the host beneficially by stimulating intestinal fermentation. The present meta-regressions support the capacity of RS type 2 to decrease hindgut pH, shift the fermentation profile to more propionate in the mid-colon and to support growth of lactic acid-producing bacteria in the distal hindgut. Increasing the RS level in pig's diet may be used to stabilize the intestinal homeostasis especially post-weaning and in the early growing period.

Introduction

Starchy feedstuffs represent the largest fraction of pig diets. However, dietary starches from different sources are digested at different rates and to different extents depending on the

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source of starch, feed processing and animal related factors (Giuberti et al., 2015). The rate, extent and gastrointestinal site where the starch is degraded will cause different physiological effects related to nutrient absorption, metabolic response and pig performance (Regmi et al., 2011; Haenen et al., 2013; Metzler-Zebeli et al., 2015a and 2015b). The starch digestibility in feedstuffs ranges from rapidly digestible to resistant to α-amylase digestion in the small intestine. The RS fraction is regarded as a type of dietary fiber and has received substantial attention in human (Birt et al., 2013) and pig nutrition (Giuberti et al., 2015). As it escapes digestion in the small intestine, RS serves as substrate for fermentation in the hindgut and has been associated with increased digesta mass, short-chain fatty acids (SCFA) production and stimulation of amylolytic and butyrogenic bacteria (Birt et al., 2013). Despite potential health benefits mediated via the action of SCFA (especially butyrate) such as ameliorated intestinal integrity and immunity, the dietary RS fraction has a controversial status in pig nutrition due to its potential negative impact on energetic efficiency and hence animal growth (Giuberti et al., 2015). Like other types of dietary fiber, RS may exert prebiotic effects and may be considered a functional dietary ingredient rather than an energy feedstuff (Bird et al., 2009; Nielsen et al., 2014). As such, RS may contribute to an improved intestinal homeostasis and disease resistance, thereby supporting growth performance in weaned and growing pigs. Yet, reported effects of RS on fermentation metabolites, such as SCFA, in pigs are inconsistent (Bird et al., 2009; Regmi et al., 2011; Nielsen et al., 2014) which may be associated with factors like source and inclusion level of RS, feed composition and the age of the animal. Currently, five different types of RS have been described (Birt et al., 2013), whereby physically inaccessible starch (RS1; e.g. intact cereal and seed grains), resistant granules (RS2; e.g. raw potatoes, high-amylose cereals (maize, barley)), and retrograded starch (RS3) are the most relevant RS types in pig diets (Giuberti et al., 2015). RS1 and RS2 are mainly found in unprocessed cereal grains, legumes and tubers, whereas RS3 forms in heat-processed feedstuffs (Giuberti et al., 2015).

Collectively, more research evidence is available for RS2 effects on gut microbial metabolites and bacteria in pigs than for the other RS types from the literature. Therefore, the primary objective of the present meta-analysis was to quantify the effect of dietary RS2 on intestinal fermentation metabolites, pH and certain key bacterial taxa for intestinal health in weaned, growing and finishing pigs. We used a meta-analytical approach, because, opposite to qualitative reviews, meta-analyses can consider changes in the direct (type and dose) and indirect factors (e.g. age of the animal) influencing starch digestion, fermentation metabolites and pH in pig's gastrointestinal tract which can cause varying results across research studies (Sales, 2014). In that way, the overall treatment effect across studies is generalized (Charbonneau et al., 2006). Effects of RS2 on apparent total tract digestibility (ATTD) were further examined for the selected research articles from which gut microbial data were extracted. Moreover, this meta-analysis aimed to derive recommendations for dietary RS levels from RS2 sources leading to physiological effects in pigs.

Material and methods

Literature search

The public search generators PubMed, Google Scholar, Web of Science (Core collections), and Scopus were used to search for literature. Research articles which investigated the effect of RS2 from purified or natural sources on the bacterial abundance and intestinal fermentation and were published in scientific journals between the years 2000 and February 2017 were considered for data extraction. Adequate articles were identified by applying the following search terms in different combinations: RS, high-amylose starch, slowly digestible starch, pig, piglet, swine, gastrointestinal tract and individual segments, gut/intestine, fermentation, microbial metabolites, total and individual SCFA (or volatile fatty acids), lactic acid, neutral and anionic forms of fermentation acids, bacteria, microbiota, microflora and microbiome.

Construction of database

The construction of the database was similar to that previously described (Metzler-Zebeli et al., 2017). Of the 35 identified articles, 24 were eligible for the present meta-analysis by meeting a sufficient number of quality criteria (Supplementary Material Table S1). Quality assessment criteria included information about dietary composition, RS level and source (purified concentrate or natural source), details on pig (breed, age, BW, age, sex, production stage), housing condition, number of pigs within treatment groups, duration of the experimental period, experimental design including randomization of treatment groups, description of statistical analysis, and intra-study error (if standard deviation was provided, it was converted into standard error), as well as bacterial abundances, microbial metabolites (i.e. SCFA and lactate) and pH values in digesta of stomach, ileum, cecum, proximal, mid and distal colon and feces (rectum). In addition, average daily feed intake (ADFI), average daily weight gain (ADG) and ATTD data were extracted. Only *in vivo* studies were included in the present meta-analysis. The main information extracted from each research article is presented in Table 1. When the dietary concentration of RS was not provided, the RS amount in the diet was estimated using published values for similar RS2 sources. In general, different analytical methodologies, including different *in vitro* α-amylase digestion approaches (e.g. Champ, 1992; Morales *et al.*, 1997) or total starch and RS assay kits (e.g. Megazyme assay kits), the official AOAC method (2002), and the determination of RS according to Englyst and Cummings (1984), were applied to determine the dietary RS concentration. Or the dietary RS content was calculated by using the manufacturer's specifications in the various research studies.

In order to consider maturational changes from weaned to finisher pigs, pig's BW at the beginning of the experiment (start BW) was used as an additional predictor variable. Likewise, the

length of the experimental period was considered as additional predictor variable because the microbial response to a dietary treatment may change over time.

From the original dataset including all studies, sub-data sets for the individual dependent variable categories were compiled, that is, one sub-dataset each for bacterial abundances, pH values, microbial metabolites including SCFA and lactate, as well as for performance-related variables such as ADG, ADFI and ATTD. Data for individual SCFA were analyzed as concentrations and molar proportions of total SCFA.

Three studies were set as minimum requirement to quantify the combined effect size (Lipsey and Wilson, 2001), together with a minimum of 10 single observations (treatment means) per dependent variable as well as the respective SEM of each variable. Only dependent variables which met these minimum requirements will be presented.

Absolute bacterial abundances were extracted from studies using quantitative PCR($n = 7$), terminal-fragment-length polymorphism ($n = 1$) and culturing ($n = 2$). Although being different methods of quantification, $\log_{10}$ colony forming units (CFU) were previously shown to highly correlate (0.979 to 0.998) to $\log_{10}$ gene copy numbers per gram of sample (Hein et al., 2001). In applying this correlation, we expressed the available bacteria data from PCR-based and culturing-based approaches in $\log_{10}$ gene copies per gram intestinal digesta or feces. Nevertheless, it needs to be kept in mind that CFU and quantitative PCR-based amplification of short sequences of the 16S rRNA gene, are different analytical approaches. Therefore, present regression results should be regarded as an approximation. If provided on DM basis, microbial metabolites and bacterial abundances were converted to fresh matter basis.

Data analysis

Descriptive statistics on predictor (i.e. dietary RS level, start BW and duration of the experimental period) and dependent variables (i.e. bacteria, microbial metabolites, pH, performance and digestibility) was performed using the MEANS procedure of SAS (SAS Institute Inc., version 9.4) similar to Metzler-Zebeli et al. (2017). Mixed modeling of each dependent variable was performed using the MIXED procedure as previously described (Metzler-Zebeli et al., 2017). The slope and intercept by study, start BW, duration of experiment and dietary RS content were included as random effects and an unstructured variance-covariance matrix (type = UN) was used to avoid a positive correlation between intercepts and slopes (St-Pierre, 2001). Breed and sex were initially included in the model but omitted in the final model as non-influential. By weighing the dependent variable by the inverse of its squared SEM (SEM of treatment means from single studies), the unequal variance among studies was taken into account. In order to test quadratic relationships between the dietary RS level and dependent variables, its squared term was included in the model using variance components (TYPE = VC) as variance-covariance matrix. However, quadratic relationships were not detectable. Data were visualized using the GPLOT procedure. Estimates, RMSE and R^2 were computed and used to evaluate the goodness of fit. Significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.10$.

Backward elimination analysis (Metzler-Zebeli et al., 2017) was used to obtain a more precise prediction of influencing factors on dependent variables that were influenced by the dietary RS level. This allowed for the simultaneous evaluation of the predictor variables dietary RS level, squared dietary RS level, start BW and length of the experimental period on the response variables. Model overparameterization was limited by allowing for a variance inflation factor of <10 (which assumes no significant multicollinearity among predictor variables tested) for every continuous independent variable tested.

Results

Database description

The RS2 originated mainly from corn, potato, pea, barley and tapioca (Supplementary Material Table S1). In the majority of studies included, diets were semi-purified with the test starch as the principal starchy component. The results of the descriptive statistics for the predictor and dependent variables are presented in Table 1. The mean and median dietary RS levels amounted to 28.8% and 23.0% of the diet (DM basis), respectively, with dietary inclusion levels ranging from 0% to 78% RS (Table 1). The BW of pigs at the beginning of the experimental period ranged between 4.6 and 105 kg with a mean and median BW of 23.7 and 30.4 kg, respectively. Pigs were fed the test diets between 7 and 100 days with a mean and median duration of the experimental period of 28.7 and 21 days, respectively. Pigs were often restrictively fed; therefore, the impact of RS2 on ADFI and ADG was not further assessed. The ranges of the predictor variables (dietary RS content, BW of the pigs at the start of the experimental period and the duration of the experimental period) in the sub-data sets for nutrient digestibility, gastrointestinal pH, microbial metabolites and bacterial counts are provided in Supplementary Material Table S2.

Resistant starch type 2 effect on intestinal and fecal pH

Established relationships between dietary RS level and intestinal and fecal pH showed that the lowering effect of RS on luminal pH became stronger from the ileum to the distal segments of the large intestine and feces. In fact, increasing RS levels tended to decrease the ileal pH ($R^2 = 0.16$; $P < 0.10$) and decreased the pH of digesta in cecum ($R^2 = 0.19$; $P < 0.05$), proximal, mid and distal colon, and feces ($R^2 = 0.37$ to 0.81; $P < 0.001$; Figure 1). According to the present equations, the diet would need to comprise 30% and 20% RS to decrease the ileal and cecal pH by 0.2 units, respectively, whereas a dietary amount of 15% would lower the pH in the proximal, mid and distal colon, and feces by 0.2, 0.6, 0.4 and 0.6 units, respectively.

Resistant starch type 2 effect on intestinal and fecal microbial metabolite concentrations

Sufficient information was available for SCFA, whereas the available information for lactate did not meet the minimum

Table 1 *Descriptive statistics for predictor and response variables for resistant starch (RS) type 2 effects in pigs*[1]

Variable	$n_{studies}$	n_{treat}	Mean	SEM	Median	Minimum	Maximum
Predictor variables							
Dietary RS (%)	24	67	28.8	3.13	23.0	0	78.0
Start BW (kg)	24	67	30.4	3.16	23.7	4.6	105.0
Experimental period (days)	24	67	28.7	2.98	21.0	7	100
Response variables							
Ileum							
pH	5	18	7.1	0.10	7.1	6.6	8.1
Total SCFA (μmol/g)	5	16	19.2	3.09	15.0	4.2	50.2
Cecum							
pH	8	30	6.1	0.09	6.2	5.2	6.8
Total SCFA (μmol/g)	7	28	84.3	6.95	90.2	27	160
Acetate (μmol/g)	7	25	45.4	4.03	42.9	12	86.4
Propionate (μmol/g)	7	25	23.5	2.62	20.4	9.	48.0
Butyrate (μmol/g)	7	25	9.5	1.30	8.8	2.0	20.8
Iso-butyrate (μmol/g)	3	13	0.37	0.11	0.14	0	1.03
Valerate (μmol/g)	3	13	1.7	0.20	1.4	0.8	3
Iso-valerate (μmol/g)	3	13	0.45	0.11	0.32	0	1.10
Proximal colon							
pH	10	30	6.0	0.09	6.1	5.2	6.8
Total SCFA (μmol/g)	9	28	81.6	8.2	82.0	29.5	170.0
Acetate (μmol/g)	7	21	37.9	4.38	44.8	7.0	61.4
Propionate (μmol/g)	7	21	14.9	2.3	15.5	4	36.4
Butyrate (μmol/g)	7	21	8.3	1.17	8.8	0.3	16.3
Mid-colon							
pH	4	15	6.5	0.17	6.7	5.4	7.2
Total SCFA (μmol/g)	3	10	62.1	16.89	59.4	3.0	175
Acetate (μmol/g)	3	10	33.3	8.62	33.8	2.0	84
Propionate (μmol/g)	3	10	17.4	4.54	15.9	1.0	50.75
Butyrate (μmol/g)	3	10	9.2	3.05	8.0	0.5	31.5
Total bacteria (log_{10} gene copies/g)	3	10	8.8	0.37	9.1	6.7	10.5
Distal colon							
pH	5	17	6.3	0.13	6.4	5.3	7.0
Total SCFA (μmol/g)	5	17	62.0	8.32	77.2	2.0	114.0
Acetate (μmol/g)	3	10	23.5	5.32	28.2	1.0	45.6
Propionate (μmol/g)	3	10	10.9	2.29	12.3	1.0	21.6
Butyrate (μmol/g)	3	10	5.	1.18	6.5	0.3	10
Feces							
pH	5	17	6.3	0.20	6.3	5.1	7.5
Total SCFA (μmol/g)	7	18	65.7	14.45	43.5	35.8	164.7
Acetate (μmol/g)	6	16	25.4	6.23	16.7	19.7	226.0
Propionate (μmol/g)	6	16	25.8	8.19	9.8	6.6	90.0
Butyrate (μmol/g)	6	18	9.7	1.11	8.2	4.7	136.0
Lactobacilli (log_{10} gene copies/g)	4	12	6.8	0.35	6.2	5.6	8.6
Bifidobacteria (log_{10} gene copies/g)	4	12	6.6	0.40	6.2	5.1	9.0
Enterobacteriaceae (log_{10} gene copies/g)	3	10	8.3	0.33	8.1	6.9	10.3
Performance and digestibility							
ADFI (g)	9	36	961	118.7	489	141	2748
ADG (g)	10	36	415	42.1	311	55	762
ATTD of DM (%)	5	16	86.1	2.52	90.0	73.2	97.6
ATTD of CP (%)	7	19	80.3	2.21	78.4	59.0	94.0
ATTD of starch (%)	6	16	99.0	0.43	99.9	93.7	100.0

ADFI = average daily feed intake; ADG = average daily weight gain; ATTD = apparent total tract digestibility; CI = confidence interval; n_{treat} = number of treatment means included; SCFA = short-chain fatty acids.
[1]Separate data sets for response variables pH, short-chain fatty acids, bacteria and performance/digestibility were built.

requirement to quantify the combined effect size. According to the present regressions, increasing the dietary level of RS did not affect total and individual SCFA concentrations in ileal, cecal and colonic digesta and feces (Supplementary Material Table S3). When comparing the molar proportions of the main SCFAs acetate, propionate and butyrate,

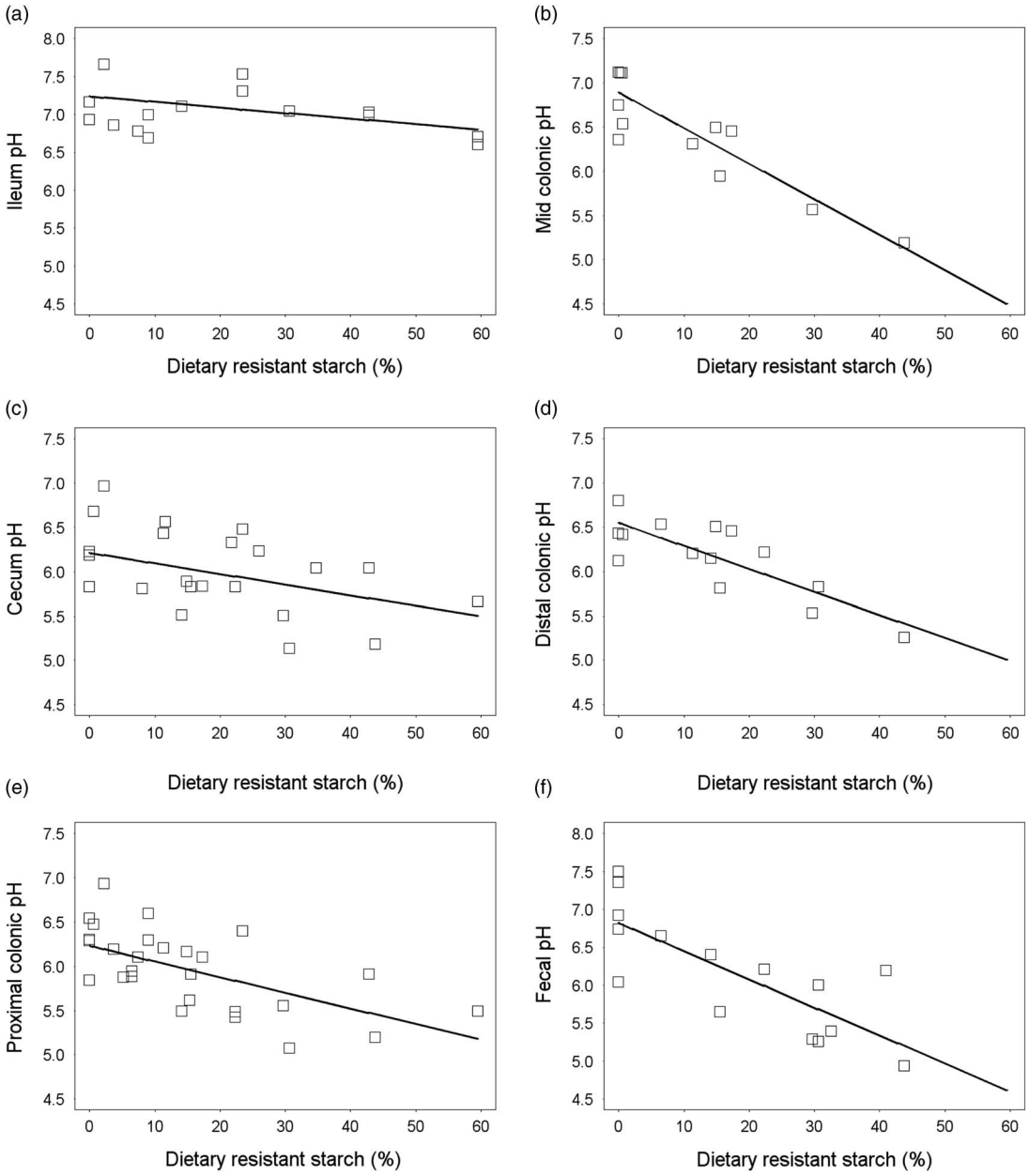

Figure 1 Linear relationships between dietary resistant starch type 2 (RS2) level and intestinal pH in pigs. Best-fit linear model of luminal pH (Y) in response to dietary RS2 level (X) (a) ileum; (b) cecum; (c) proximal colon; (d) mid-colon; (e) distal colon; and (f) feces.

relationships between the dietary RS level and the SCFA profiles became apparent. As such, regressions showed that increasing dietary RS levels decreased the molar proportion of acetate and butyrate but increased that of propionate in mid-colonic digesta ($R^2 = 0.44$ to 0.57; $P < 0.05$; Table 2). In distal colonic digesta, only the molar proportion of butyrate was negatively related to increasing dietary RS levels ($R^2 = 0.50$; $P = 0.022$). A minimum of 20% of dietary RS would be needed to elevate the molar propionate proportion by 5% in mid-colonic digesta.

Resistant starch type 2 effect on bacterial abundances

Sufficient information was available for total bacteria in colonic digesta and for lactobacilli, bifidobacteria and *Enterobacteriaceae* in feces (Table 3). According to the present regressions, increasing the RS level did not affect the total bacterial number in colonic digesta, whereas it promoted the presence of lactobacilli ($R^2 = 0.46$, $P < 0.001$) and bifidobacteria ($R^2 = 0.52$, $P < 0.001$) in feces. To achieve a physiological relevant increase in fecal lactobacilli and bifidobacteria, a minimum of 10% RS in the diet may be required.

Resistant starch type 2 effect on apparent total tract digestibility

The ATTD of DM, CP and starch was not modified by increasing dietary RS levels (Table 4).

Table 2 *Prediction of molar proportions of individual short-chain fatty acids (SCFA mol/100 mol) in cecal and colonic digesta and feces as affected by dietary resistant starch content (%) in pigs*

	Parameter estimates				Model statistics		
Response variable (*Y*)	Intercept	$SEM_{Intercept}$	Slope	SEM_{Slope}	RMSE	R^2	*P* value
Cecum							
Acetate	57.69	1.96	−0.068	0.068	5.886	0.05	0.327
Propionate	28.50	1.77	−0.005	0.061	5.318	0.00	0.931
Butyrate	11.74	1.00	−0.033	0.034	2.984	0.04	0.340
Proximal colon							
Acetate	59.01	4.29	−0.314	0.189	13.945	0.13	0.114
Propionate	21.41	3.29	0.048	0.145	10.689	0.01	0.742
Butyrate	10.98	1.18	0.036	0.052	3.846	0.02	0.502
Mid-colon							
Acetate	59.29	2.14	−0.356	0.109	4.789	0.57	0.012
Propionate	26.68	1.47	0.241	0.075	3.288	0.56	0.012
Butyrate	15.36	1.22	−0.157	0.062	2.723	0.44	0.035
Distal colon							
Acetate	57.09	2.66	−0.150	0.132	5.660	0.14	0.286
Propionate	29.96	4.00	−0.025	0.197	8.492	0.00	0.901
Butyrate	13.76	1.10	−0.154	0.054	2.335	0.50	0.022
Feces							
Acetate	47.24	6.58	0.115	0.267	19.061	0.01	0.674
Propionate	22.20	7.23	0.070	0.293	20.932	0.00	0.814
Butyrate	9.12	1.40	0.021	0.057	4.063	0.01	0.721

Table 3 *Prediction of absolute bacterial abundance (log_{10} gene copies/g) in colonic digesta and feces as affected by dietary resistant starch content (%) in pigs*

	Parameter estimates				Model statistics		
Response variable (*Y*)	Intercept	$SEM_{Intercept}$	Slope	SEM_{Slope}	RMSE	R^2	*P* value
Mid-colon							
Total bacteria	8.36	0.60	0.015	0.019	1.106	0.08	0.427
Feces							
Lactobacilli	5.81	0.46	0.047	0.015	0.926	0.46	0.008
Bifidobacteria	5.52	0.42	0.046	0.014	0.950	0.52	0.008
Enterobacteriaceae	8.46	0.71	0.005	0.021	1.208	0.01	0.824

Table 4 *Prediction of apparent total tract digestibility of nutrients as affected by dietary resistant starch content (%) in pigs*

	Parameter estimates				Model statistics		
Response variable (*Y*)	Intercept	$SEM_{Intercept}$	Slope	SEM_{Slope}	RMSE	R^2	*P* value
ATTD of DM (%)	84.0	3.28	0.109	0.106	10.03	0.07	0.322
ATTD of CP (%)	82.8	2.56	−0.185	0.109	9.11	0.14	0.109
ATTD of starch (%)	98.8	0.63	0.017	0.029	1.82	0.03	0.558

ATTD = coefficient of total tract apparent digestibility.

Backward elimination analysis

Backward elimination analysis showed that the starting BW of pigs and length of the experimental period influenced the effect of dietary RS2 on the response variables (Table 5). The response variables cecal pH, distal colonic pH and mid-colonic acetate proportion were mainly affected by the increasing dietary RS level ($R^2 = 0.19$ to 0.65; $P < 0.05$). According to the squared effect of the RS level, the response

Table 5 *Best-fit equations showing the coefficients of microbial response variables and average daily weight gain in relation to increasing dietary resistant starch (RS) content, pig's starting BW and length of the experimental period using backward elimination*

		Parameter estimates				Model statistics			
Response variable (*Y*)	Factor (*X*)	Intercept	$SEM_{Intercept}$	Slope	SEM_{Slope}	RMSE	R^2	VIF	*P* value
Fecal lactobacilli (log_{10} gene copies/g)	Experimental period (days)	5.43	0.22	0.024	0.004	0.452	0.88	1.03	<0.001
	Squared RS content (%)			0.001	0.0001			1.03	0.001
Fecal bifidobacteria (log_{10} gene copies/g)	Experimental period (days)	7.32	0.75	−0.094	0.040	0.689	0.78	1.00	0.044
	Squared RS content (%)			0.001	0.0002			1.00	<0.001
Ileal pH	Squared RS content (%)	7.21	0.09	−0.0001	0.0001	0.331	0.22	1.00	0.048
Cecal pH	RS content (%)	6.21	0.12	−0.012	0.005	0.406	0.19	1.00	0.028
Proximal colonic pH	Experimental period (days)	6.38	0.11	−0.004	0.002	0.337	0.46	1.00	0.039
	RS content (%)			−0.018	0.004			1.00	<0.001
Mid-colonic pH	Experimental period (days)	5.35	0.61	0.064	0.025	0.277	0.83	1.29	0.024
	RS content (%)			−0.033	0.006			1.29	<0.001
Distal colonic pH	RS content (%)	6.57	0.09	−0.027	0.005	0.272	0.65	1.00	<0.001
Fecal pH	RS content (%)	6.94	0.17	−0.082	0.026	0.461	0.69	12.89	0.006
	Squared RS content (%)			0.001	0.0007			12.89	0.084
Mid-colonic acetate proportion (%)	RS content (%)	59.35	1.99	−0.362	0.102	4.457	0.61	1.00	0.007
Mid-colonic propionate proportion (%)	Start BW (kg)	59.29	11.12	−1.279	0.447	1.944	0.85	1.08	0.024
	RS content (%)			0.005	0.001			1.08	0.002
Mid-colonic butyrate proportion (%)	Start BW (kg)	−3.43	8.52	0.767	0.341	1.495	0.83	1.06	0.059
	RS content (%)			−0.165	0.035			1.06	0.002
Distal colonic butyrate proportion (%)	RS content (%)	16.00	0.62	−0.509	0.075	1.132	0.92	8.04	<0.001
	Squared RS content (%)			0.007	0.002			8.04	0.0042

VIF = variance inflation factor.

variables fecal lactobacilli and bifidobacteria, ileal pH and distal colonic butyrate proportion followed an asymptotic approximation with increasing RS levels ($P < 0.05$). Moreover, backward elimination showed that, besides the positive relationship with dietary RS, the duration of the experimental period increased the fecal numbers of lactobacilli ($R^2 = 0.88$; $P < 0.05$) but decreased those of bifidobacteria ($R^2 = 0.78$; $P < 0.05$). The duration of the experiment also potentiated the negative relationship between dietary RS level and proximal colonic pH ($R^2 = 0.46$; $P < 0.05$), whereas it counteracted the decline in mid-colonic pH with increasing dietary RS level ($R^2 = 0.83$; $P < 0.05$). Moreover, BW at the start of the experiment influenced mid-colonic propionate ($P < 0.05$) and mid- ($P < 0.10$) and distal colonic butyrate proportions ($P < 0.05$) in opposite directions.

Discussion

The present study aimed to systematically and statistically evaluate the capability of dietary RS2 to modulate intestinal bacterial abundances and fermentation. In particular, meta-regressions demonstrated that, with a minimum amount of 10% to 15% actual RS in the diet, RS2 can effectively decrease the luminal pH in the hindgut of pigs and promote lactic acid-producing bacteria in pig's feces. Although the extracted data covered a wide range of experimental conditions, it should be considered for the interpretation of the present regressions that most information was available for luminal pH in the cecum and proximal colon with 21 to 30 treatment comparisons. For the other response variables, often only low numbers of treatment comparisons could be extracted. Also, as indicated by the mean and median BW of pigs, present relationships are more applicable to smaller pigs.

Due to its low digestibility in the small intestine, it is generally assumed that RS2 promotes fermentation especially in the proximal regions of the large intestine (Bach Knudsen et al., 2012; Giuberti et al., 2015). As a consequence, most research data on RS2 effects on SCFA were available for the cecum, colon and feces. Fermentation data for stomach, duodenum and jejunum from research articles between the years 2000 and 2017 were scarce and only response variables in ileal digesta met our minimum requirements for inclusion in this meta-analysis. This was despite the fact that the pig harbors a highly diverse and numerous microbial community in the stomach where fermentation of carbohydrates already commences (Metzler-Zebeli et al., 2013; Motta et al., 2017).

Due to the fermentation occurring prececally, the dietary concentration of RS is one of the critical factors for the effectiveness of RS2 to modify SCFA concentrations in the hindgut of pigs. Moreover, endogenous RS levels in low-amylose cereal grains and legumes, depending on the variety, range from 1% to 20% (Birt et al., 2013; Giuberti et al., 2015) and may have masked potential RS effects if dietary levels were too low. There was only one study in which RS2 was supplemented at very low levels of 0.5% and 1% RS in powder form or as a capsule (Heo et al., 2014). In all other studies, higher RS (amylose) contents were examined, which should have been sufficient to produce detectable effects of the respective RS2 source on

intestinal parameters. Many studies utilized semi-purified diets, thereby greatly circumventing that effects may have been masked by other fermentable dietary ingredients. Using semi-purified diets though may reduce the transferability of results to whole-grain cereal and legume meal diets due to interactions among ingredients of more complex diets. It is very probable that other dietary carbohydrates (e.g. pectins, arabinoxylans, β-glucans, cellulose) modified the RS2-induced effects on intestinal SCFA, bacterial abundances and digestibility of starch in the research studies. However, those carbohydrate fractions could not be incorporated in the present meta-analysis as the chemical composition of experimental diets was poorly reported in more than half of the included research articles. Moreover, different analytical methodologies or the specification of the manufacturer that provided the starch were used to estimate the dietary RS content which may have added a certain variability with respect to the interpretation of the RS2 effects among the included research articles. With RS2 effects detectable for certain parameters (i.e. luminal pH along large intestinal segments, bacteria in feces and SCFA profiles in mid and distal colon), the slopes of the regression equations indicated that minimal dietary amounts between 10% and 20% RS are necessary to cause measurable physiological effects along the large intestine.

The intestinal pH is indicative of microbial activity and has been used as measure for intestinal health in pigs by suppressing the growth of opportunistic pathogens (Heo et al., 2014). The pH in the large intestine is largely affected by the amount of dietary fermentable carbohydrates entering the large intestine, thereby stimulating microbial activity and generation of SCFA which consequently acidify the digesta (Wang et al., 2004; Heo et al., 2014). Present meta-regressions supported this principle for RS2, showing greater acidification of digesta in the large intestine with increasing dietary RS levels. Notably, relationships between the dietary RS content and digesta pH became stronger in the distal segments of the large intestine. Due to the high degradability of RS2 sources, for example, raw potato starch and high-amylose cornstarch, fermentation intensity has been often assumed to be highest in cecum and proximal colon (Haenen et al., 2013; Sun et al., 2015). Therefore, it seems plausible that the steeper slopes for the pH decline in the mid and distal colon and feces can be partly associated with an accumulation of protons in digesta. Within a luminal pH range of 6 to 7 (Bergman, 1990), lactate and SCFA are ionized and require monocarboxylate transporters for uptake (Sepponen et al., 2007), whereas protons remain in the intestinal lumen, thereby increasing digesta acidity. In addition, proton exchangers reduce the intracellular proton load after uptake of the acid form of lactate and SCFA into enterocytes, thereby further acidifying the intestinal lumen (Collins et al., 1993; Thwaites and Anderson, 2007). Backward elimination results of luminal pH indicated that an adaptation of the microbiota may increase the production of SCFA and hence acidification in the proximal colonic digesta when pigs are fed the RS2 for a longer time period, leading to a stronger pH decline. Increased microbial degradation of RS in the proximal large intestine and a reduced substrate flow to the mid-colon may subsequently explain the positive relationship between mid-colonic pH and a longer experimental period.

There are several possible explanations for the missing relationship between dietary RS and SCFA along the large intestine and feces. As data for SCFA were provided as concentrations, they do not fully represent the total amount of SCFA produced on a daily basis (Regmi et al., 2011). Fermentable fiber increases the digesta bulk in the hindgut (Bach Knudsen et al., 1993; Pluske et al., 2007); therefore, daily production of SCFA may be still increased by the present RS2 sources but not reproducible from the data available from the individual research studies. Second, increased synthesis of SCFA stimulates their mucosal uptake which may have lowered concentrations measured in digesta (Cummings and Macfarlane, 1991). Nevertheless, meta-regressions support that dietary RS levels can decrease the molar proportion of acetate and increase that of propionate in the mid-colon, thereby supporting results from individual studies (e.g. Martinez-Puig et al., 2003). Changes in the *Lachnospiraceae*, *Clostridiaceae* and *Bacteroidaceae* families, which comprise propionate-producing bacteria (Reichardt et al., 2014), have been reported in pigs fed RS2 (Sun et al., 2015 and 2016). Enhanced generation of propionate may have an important impact on host physiology by regulating gene expression and as signaling molecule (Louis and Flint, 2017). Propionate exerts anti-inflammatory properties at the intestinal mucosa and, after absorption, contributes to gluconeogenesis in the liver and can promote satiety (Morrison and Preston, 2016). Although some studies reported a stimulating effect of RS2 on butyrate fermentation (Mentschel and Claus, 2003), our findings of negative relationships between the butyrate proportion and dietary RS level in the mid and distal colon were rather unexpected. Maturational processes within the intestinal microbiota may have influenced the outcome of the various individual studies as indicated by the best-fit equations with decreasing propionate and increasing butyrate proportion with increasing starting BW. Also, the aforementioned discrepancy between SCFA produced on a daily basis and those measured per gram digesta should be considered in this context.

Overall, the three dominant phyla of the porcine microbiota, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, comprise important starch-degrading bacterial species (Haenen et al., 2013; Sun et al., 2015 and 2016). Amylase and pullulanase activities have been reported, among others, for lactobacilli, bifidobacteria, *Microbacterium*, *Turicibacter*, *Blautia*, *Ruminococcus*, especially *Ruminococcus bromii*-phylotypes, and *Bacteroides* (Louis et al., 2007; Sun et al., 2015 and 2016; Louis and Flint, 2017). Other bacteria, such as *Faecalibacterium*, *Coprococcus, Megasphaera* and *Mitsuokella*, were shown to prosper on RS2-containing diets (Sun et al., 2015 and 2016), probably via cross-feeding of fermentation metabolites. Particularly, cross-feeding of lactate and succinate to propionate-producing bacteria

(Louis and Flint, 2017) may explain the relationships of dietary RS level with colonic SCFA profiles. The extractable data from the research articles that met the eligibility criteria and the minimum requirement to quantify the combined effect size, however, only allowed for assessing relationships between dietary RS levels and total bacterial numbers in colonic digesta and the three best investigated bacterial groups lactobacilli, bifidobacteria and *Enterobacteriaceae* in feces. The missing relationship between RS level and total bacteria in the colon may confirm that RS2-associated alterations in the overall bacterial community structure and diversity may be expected rather than changes in their total numbers as reported in some individual studies (Sun et al., 2015). Current meta-regressions further support the promotion of fecal lactobacilli and bifidobacteria by dietary RS2 sources in pigs. High intestinal abundance of lactobacilli and bifidobacteria has been generally associated with intestinal health, as these genera can contribute to suppress the growth of opportunistic pathogens (Schmidt et al., 2010; O'Shea *et al*., 2012; Yang et al., 2015; Wang et al., 2016) and exert immunomodulatory effects (Kandasamy et al., 2014; Kamiya et al., 2016). Although these relationships were obtained for feces, they likely reflect the conditions in the distal hindgut. Although belonging to pig's commensal microbiota, *Enterobacteriaceae* were mostly investigated because they comprise opportunistic pathogens, such as *Salmonella* or enterotoxigenic *Escherichia coli* (Fairbrother et al., 2005). As pH-sensible bacteria, *Enterobacteriaceae* abundance may have been decreased by the RS-related decrease in luminal pH in the distal large intestine, which was not confirmed by the present regression analysis. The RS concentration in digesta consecutively decreases along the large intestine, which may lead to different effects in the modulation of microbial taxa from the proximal to distal large intestine (Haenen et al., 2013). Therefore, relationships obtained for the distal segments in the current meta-analysis should not be used to predict relationships in the cecum or proximal colon. Moreover, data from culturing and PCR-approaches were combined which should be considered when interpreting the present relationships for colonic digesta and feces. By showing a squared effect of dietary RS level, backward elimination confirmed that a minimum dietary RS amount is necessary to enhance lactobacilli and bifidobacterial numbers. Best-fit equations also suggested short-term and long-term effects of RS2, which were opposite for lactobacilli and bifidobacteria. As both genera comprise starch-degraders, the decrease in bifidobacteria over time may be related to a competition with other starch-degrading bacteria, such as amylolytic lactobacilli, and to age-related changes in the bacterial community post-weaning (Yang et al., 2015; Bian et al., 2016).

Evidence exists that high dietary RS levels negatively impact ileal DM and energy digestibility (Giuberti et al., 2015). As the focus of the present meta-analysis was on intestinal bacterial action and not on nutrient digestibility, data for apparent ileal digestibility of DM, energy and nutrients that could be extracted were limited and very likely not representative. Results support that the ATTD of starch is almost complete which can be clearly explained by the high fermentability of the various RS2 sources. However, less energy can be gained from intestinal fermentation than from host enzymatic starch digestion (Bach Knudsen et al., 1993), leading to the often reported depression in growth performance with increasing dietary RS intake (Giuberti et al., 2015).

Conclusion

According to the present meta-regressions, dietary RS2 sources can effectively decrease the luminal pH in the large intestine, especially in the more distal regions, of pigs, which may be helpful to suppress the growth of opportunistic pathogens. Moreover, relationships supported that lactic acid-producing bacteria such as lactobacilli and bifidobacteria in feces and SCFA generation, especially propionate in the mid-colon, can be promoted by increasing dietary RS levels. However, present estimations indicated that, in order to achieve physiologically relevant changes, the actual dietary RS concentration should surpass 10% to 15%. Moreover, it needs to be considered that for many response variables, only low numbers of treatment comparisons were available and no differentiation between different RS2 sources could be made.

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Declaration of interest

The authors declare no potential conflict of interest.

Ethics statement

The present meta-analyses used statistical regression methods to analyze previously recorded data and did not require ethical approval.

Software and data repository resources

Data were not deposited in an official repository.

Supplementary material

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