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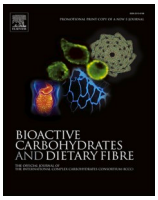

The prebiotic potential of RS-3 preparations for pre- and post-weaning piglets

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ABSTRACT

Piglets undergo stress during the weaning period, resulting in an imbalance in gut microbial composition and activity, and potentially post-weaning diarrhoea. Supporting maturation of gut microbiota is a strategy preparing piglets for weaning, and could be achieved by providing dietary fibres such as resistant starch (RS) in the creep feed. We investigated the prebiotic potential of four well-characterised retrograded starches (RS-3 preparations) for pre- and post-weaning piglets. These RS-3 preparations were *in vitro* fermented using pooled faecal inoculum of 3-week-old (pre-weaning) and 7-week-old (post-weaning) piglets. RS-3 preparations containing a high amount of digestible starch (≥ 75 %) were fully fermented by both faecal inocula within 48 h. Such substrates generated mostly butyrate when fermented by pre-weaning piglet faecal inoculum, whereas mostly propionate was found during fermentation by post-weaning inoculum. Bacterial genera *Prevotella* and *Megasphaera* increased in relative abundance after fermentation by both inocula, whereas *Streptococcus* and *Selenomonas* increased in relative abundance only when fermented by pre-weaning or post-weaning piglet faecal inocula, respectively. Highly resistant or so-called intrinsic RS-3 (≥ 80 % RS) was hardly degraded by pre-weaning inoculum, whereas it was slowly fermented by post-weaning piglet faecal microbiota, leading to an increase in relative abundance of specific *Prevotella* and *Roseburia* phylotypes (amplicon sequence variants). This study shows that partially resistant RS-3 preparations might be beneficial dietary fibres for pre-weaning piglets by promoting short-chain fatty acids production, while intrinsic RS-3 was hardly fermentable. Intrinsic RS-3 substrates might have prebiotic potential for post-weaning piglets by stimulating potential health-beneficial *Prevotella* and *Roseburia*.

1. Introduction

In commercial pig husbandry, piglets are weaned around 3–4 weeks of age (Gresse et al., 2017), which is much younger than the age of 15–22 weeks in nature (Jensen & Stangel, 1992). This abrupt and early weaning causes stress, since piglets are removed from the sow and need to adjust from an easily-digestible liquid milk diet to a more complex plant-based solid feed (Guevarra et al., 2019). Due to this abrupt change, feed intake usually decreases, resulting in undernutrition and weight loss (Lallès et al., 2004). In addition, weaning could possibly cause a disbalance in gut microbiota composition, which gives the opportunity for enterotoxigenic *E. coli* (ETEC) to proliferate resulting in post-weaning diarrhoea (PWD), dehydration and even death (Sun & Kim, 2017). PWD can be overcome effectively by the use of antibiotics such as colistin (Rhouma, Fairbrother, Beaudry, & Letellier, 2017), or therapeutic doses of zinc oxide (Bonetti, Tugnoli, Piva, & Grilli, 2021). However, the preventive use of antibiotics and zinc oxide is forbidden in the EU ("Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC,") due to increasing antibiotic

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resistance and environmental impact. Potential alternatives to decrease the post-weaning stress and potential incidence of PWD comprise nutritional strategies (Huting, Middelkoop, Guan, & Molist, 2021) such as inclusion of probiotics (Bogere, Choi, & Heo, 2019), prebiotics (Bai et al., 2021; Choudhury et al., 2021; de Vries et al., 2020; Van Hees et al., 2021), digestive enzyme cocktails (Slupecka et al., 2012) or environmental changes such as enriched housing (Saladrigas-Garcia et al., 2021; Wen et al., 2021).

The gut microbiota composition of piglets changes dramatically during the first weeks of life, when switching from sow's milk to a plant-based starter diet. Relative abundance of e.g. *Bacteroidaceae* and *Enterobacteriaceae* were shown to decline when changing to a starter diet, whereas *Lactobacillaceae*, *Ruminococcaceae*, *Veillonellaceae* and *Prevotellaceae* were shown to increase (Frese, Parker, Calvert, & Mills, 2015). Healthy 2-week post-weaning piglets were shown to have higher relative abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae*, compared to diarrheic piglets (Dou et al., 2017). Pre-weaning supplementation of prebiotics, such as dietary fibres, has been shown to support faster maturation of the gut microbiota composition and to have positive effects on intestinal barrier function and production of short-chain fatty acids (SCFAs) (Huting et al., 2021), compared to piglets not receiving such dietary fibres (Choudhury et al., 2021). A wide variety of dietary fibres have been tested *in vivo* for their prebiotic potential pre-weaning, such as inulin (Li et al., 2018), fructo-oligosaccharides (Schokker et al., 2018), galacto-oligosaccharides (GOS) (Tian, Wang, Yu, Wang, & Zhu, 2018), arabinoxylans (Van Hees et al., 2021) and β-glucans (de Vries et al., 2020) or a combination of dietary fibres (Choudhury et al., 2021; Wu et al., 2020). It has also been suggested that resistant starch as a prebiotic may reduce the incidence of PWD and associated mortality (Tan, Beltranena, & Zijlstra, 2021).

Starch is the main energy source of pigs and, depending on the source, is fully digested in the small intestine of growing pigs (Martens et al., 2019) or partially fermented in the colon (Van Erp, De Vries, Van Kempen, Den Hartog, & Gerrits, 2020). Based on the Englyst *in vitro* digestion model, digestion kinetics can divide starch into three different fractions: rapidly digestible starch, slowly digestible starch and resistant starch (Englyst, Kingman, & Cummings, 1992). Resistant starch is the starch fraction that escapes digestion by host enzymes within the upper gastro-intestinal tract (GIT), to be fermented in the ileum or colon of pigs. Although total tract digestibility of starch as measured in faeces is mostly complete in growing pigs, ileal digestibility differs depending on, among others, the botanical source (Wiseman, 2006), processing (Martens et al., 2019) and the amount of starch supplemented (Bhandari, Nyachoti, & Krause, 2009). Pancreatic α-amylase activity in piglets increases with age (Wiseman, Pickard, & Zarkadas, 2000) with a 6-fold increase in 3-week-old piglets compared to new-borns (Pond, Snook, McNeill, Snyder, & Stillings, 1971). It was also shown that amylase activity further increased until similar activities were observed between 10 and 14 week-old pigs and 6-month-old pigs (Westrom, Ohlsson, & Karlsson, 1987). Consequently, starch hydrolysis within the small intestine of piglets 0–10 days post-weaning was lower, compared to starch hydrolysis 14–28 days post-weaning, that was similar to growing pigs and sows (Bach Knudsen, Hedemann, & Laerke, 2012). This indicates that the proportion of digestible starch as defined by Englyst et al., 1992 reaching the ileum or colon depends on the age of the piglet, and consequently a proportion of digestible starch may be subject to fermentation in young piglets.

Different types of resistant starch exist of which RS-1 (starch locked due to a cell-wall matrix, such as in whole cereals) and RS-2 (native granules, such as high-amylose maize starch) (Birt et al., 2013) are quite common in the pig diet (Aumiller, Mosenthin, & Weiss, 2015). RS-3 refers to retrograded starch (Birt et al., 2013) and can be prepared after starch gelatinisation and cooling to recrystallise the starch molecules. RS-3 preparations, made by crystallisation of α-1,4 glucans in a controlled way, may contain an *in vitro* digestible fraction, depending on physico-chemical parameters such as crystal type, chain length and molecular weight distribution (Klostermann et al., 2021). Some RS-3 preparations made from short α-1,4 glucans were shown to be highly resistant to digestion (80–95 % RS) (Cai & Shi, 2013; Klostermann et al., 2021), and are therefore called "intrinsic RS-3".

So far, no studies investigated the prebiotic potential of RS-3 (preparations) for pre- and post-weaning piglets. Raw potato starch (RS-2) was shown to increase colonic lactobacilli and *Bacteroides* in nursing piglets (Bhandari et al., 2009), whereas high-amylose maize starches were shown to increase caecal and colonic *Bifidobacterium* (Fouhse, Ganzle, Regmi, van Kempen, & Zijlstra, 2015) and proximal colonic lactobacilli and bifidobacteria in nursing piglets (Bird, Vuaran, Brown, & Topping, 2007). Nevertheless, in an *in vitro* study it was shown that faecal microbiota of pre-weaning piglets fermented raw potato starch (RS-2) poorly, whereas gelatinised potato starch was fermented properly (Bauer, Williams, Voigt, Mosenthin, & Verstegen, 2001). In contrast, in the same study, faecal microbiota obtained from adult pigs was able to ferment both raw and gelatinised potato starch (Bauer et al., 2001). In adult pigs it was shown that a commercial RS-3 ($\geq$ 50 % RS) was fully fermented in the caecum and positively influenced the SCFAs production and microbiota composition (Haenen et al., 2013). However, the fermentability of RS-3 preparations by faecal inoculum of pre- and post-weaning piglets and the effects of such substrates on microbiota composition are still unknown.

In this study, we therefore investigated the prebiotic potential of four RS-3 preparations varying in physico-chemical characteristics and *in vitro* digestibility for pre- and post-weaning piglets. Faeces of pre- (3-week-old) and post- (7-week-old) weaning piglets was collected, pooled per age and used as inoculum for *in vitro* fermentation. The fermentation kinetics were followed over time by quantifying the remaining soluble and insoluble starch fractions and SCFAs generated. Possible changes in microbiota composition were evaluated after 16S ribosomal (rRNA) gene sequencing, and the starch morphology during *in vitro* fermentation was visualised using scanning electron microscopy.

2. Materials and methods

2.1. Materials

All medium components used for the *in vitro* fermentation were obtained from Tritium Microbiologie (Eindhoven, The Netherlands). L-cysteine-hydrochloride, 2-(N-morpholino) ethanesulfonic acid (MES) and soluble potato starch (SPS) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

2.1.1. RS-3 preparations

RS-3 preparations were prepared by crystallising α-1,4 linked glucans obtained from either debranched highly branched starch, enzymatically modified from potato (HBPS, molecular weight (Mw) ±100 kDa, 8 % branch points, AVEBE (Veendam, The Netherlands)) (P14-A) or enzymatic synthesis (N18-A, N18-B, N32-B) as reported previously (Klostermann et al., 2021). The physico-chemical characteristics and *in vitro* digestibility of the RS-3 preparations are summarised in Table 1 and discussed in the results section.

2.1.2. Faecal slurry

Faecal samples were obtained from cross-bred piglets (Danbred x Piétrain; INNSOLPIG, Aalter, Belgium) of mixed gender (gilts and castrated boars) and normal weight that had not been exposed to antibiotics. From the age of 6 days piglets had ad-libitum availability to an experimental solid creep feed (Prestarter diet (Table S1), Nuscience Group, Apeldoorn, The Netherlands) until weaning at 19 ± 2 days of age. From weaning until 40 days old, the piglets were fed an experimental weaning diet (Table S1, Nuscience Group) followed by an experimental starter diet (Table S1, Nuscience Group). The diets contained a raw starch component, which may have influenced the gut

Table 1
Characteristics of RS-3 preparations and SPS used in this study, data obtained from Klostermann et al. (2021). DPn (number-based degree of polymerisation) and PI (polydispersity index) were determined using high performance size exclusion chromatography with refractive index detection (HPSEC-RI), crystal type was determined using X-ray diffraction and rapidly digestible starch (RDS, 20 min digestion), slowly digestible starch (SDS, 20–120 min digestion) and resistant starch (RS, >120 min digestion) were determined after digestion using pancreatin and amyloglucosidase.

Sample name	Reported as[a]	Crystal type	DPn	PI	RDS (%)	SDS (%)	RS (%)
SPS		–	–	–	100	–	–
P14-A	dHBPS-A	A	14	1.33	40.1 ± 5.4	44.7 ± 8.9	15.2 ± 3.6
N18-A	sG5-A	A	18	1.21	12.4 ± 9.1	0 ± 6.1	88.0 ± 3.8
N18-B	sG5-B	B	18	1.21	50.7 ± 0.4	23.4 ± 3.0	25.9 ± 3.3
N32-B	sG20-B	B	32	1.14	7.9 ± 7.6	0 ± 5.0	94.9 ± 2.7

[a] (Klostermann et al., 2021).

microbiota composition of the piglets prior to sampling the faeces. Pre-weaning faecal samples were collected from healthy piglets (3 castrated boars, 5 gilts) of different sows around 18 ± 2 days of age. Post-weaning piglet faecal samples were collected at 49 ± 2 days (3 castrated boars, 6 gilts). Seven of the piglets sampled pre-weaning were also sampled post-weaning. Faecal samples were obtained by stimulating the rectum with a sterile swap and collecting the faeces directly into a sterile 50 mL container with a filter screw cap (Greiner Bio-One CELLSTAR™ tube, Kremsmünster, Austria). The container was placed inside a pouch with a BD GasPak EZ anaerobe gas generating system with indicator (BD Diagnostics, Sparks, Maryland, USA) and transported cold, using cooling elements, to the laboratory and stored at 4 °C for maximum 24 h until processing. Equal masses of the faeces collected individually from either pre-weaning piglets or post-weaning piglets were pooled under anoxic conditions (4 % H_2, 15 % CO_2, 81 % N_2, Bactron 300, Sheldon Manufacturing, Cornelois, USA) and diluted to 25 % w/v using pre-reduced PBS-glycerol containing 10 % glycerol, 0.5 mg/L resazurin and 0.4 g/L L-cysteine-hydrochloride, according to the method of Aguirre et al. (2015). The final faecal slurries were snap-frozen using liquid nitrogen and stored at −80 °C prior to use.

2.2. In vitro batch fermentation of RS-3 preparations

The culture medium was based on Standard Ileal Efflux Medium (SIEM) (Minekus et al., 1999) with minor modifications (0.592 g/L carbohydrates) as described previously (Klostermann, Endika, Ten Cate, et al., 2023) and is referred to as mSIEM. The medium was stored overnight in an anaerobic chamber to remove the head-space oxygen. Culture medium containing 11.11 mg/mL soluble potato starch (mSIEM + SPS) was prepared as described previously (Klostermann, Endika, Ten Cate, et al., 2023). Approximately 20 mg (dry weight) of the RS-3 preparations (Table 1) were weighed in duplicate in sterile 5 mL serum bottles for each individual sampling time and stored overnight in the anaerobic chamber. For the experiment using pre-weaning piglet faecal inoculum, we incubated P14-A, N18-A, N18-B and SPS, whereas we included one more intrinsic RS-3 (N32-B) using post-weaning faecal inoculum. From preliminary results it was observed that N32-B was hardly fermented by pre-weaning piglet faecal microbiota (data not shown).

In the anaerobic chamber, inocula were prepared by diluting faecal slurry to 10 mg/mL faeces in mSIEM. An aliquot of 1.8 mL mSIEM or 1.8 mL mSIEM + SPS and 0.2 mL inoculum were added to the serum bottles to reach final substrate concentrations of ±10 mg/mL. Medium blanks (without additional substrate, including mSIEM carbohydrates) and substrate blanks (without inoculum) were also prepared. The serum bottles were capped with butyl rubber stoppers and incubated at 39 °C, 100 rpm for 0, 8, 24, 36 and 48 h.

At given time points, samples were collected as described previously (Klostermann, Endika, Ten Cate, et al., 2023). Briefly, the serum bottles were decapped and the contents were transferred to Safe-Lock Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 4 °C, 20,000×*g* for 10 min. The supernatant and pellet were separated and further processed. The supernatant was heated in Safe-Lock Eppendorf tubes to 100 °C, 800 rpm for 10 min in an Eppendorf shaker and stored at −20 °C until analysis. The pellet was snap-frozen using liquid nitrogen and freeze-dried.

2.3. Total starch quantification

Starch was quantified in the supernatants and freeze-dried pellets obtained after *in vitro* fermentation using the Megazyme Total Starch Kit (AA/AMG) (Megazyme, Wicklow, Ireland) according to the manufacturer's protocol and adjusted for smaller sample sizes as previously described (Klostermann, Endika, Ten Cate, et al., 2023). Freeze-dried pellets containing N18-A and N32-B were solubilised using 1.7 M NaOH, whereas freeze-dried pellets containing P14-A and N18-B were solubilised by boiling, as described previously (Klostermann, Endika, Ten Cate, et al., 2023). After appropriate neutralisation, dilution and enzymatic hydrolysis of the solubilised starch, free glucose content was analysed using the GOPOD assay kit (Megazyme) as described previously (Klostermann, Endika, Ten Cate, et al., 2023).

2.4. Short-chain fatty acids and other organic acids produced by fermentation

SCFAs, branched-chain fatty acids (BCFAs), lactic and succinic acid were analysed as described previously (Klostermann, Endika, Ten Cate, et al., 2023). Samples were diluted 5 times using Milli-Q water and centrifuged. The supernatant (10 µL injection volume) was analysed using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, California, USA) with an Aminex HPX-87H column (Bio-Rad laboratories Inc, Hercules, California, USA). Elution was performed at 0.5 mL/min and 50 °C using 50 mM sulphuric acid as eluent. Acids were detected by a refractive index detector (RI-101, Shodex, Yokohama, Japan) and a UV detector set at 210 nm (Dionex Ultimate 3000 RS variable wavelength detector). Standard curves of acetic, propionic, butyric, isobutyric, isovaleric, lactic and succinic acid were prepared (0.05–2 mg/mL) for quantification.

2.5. Scanning electron microscopy

Aliquots of 20 µL digesta of RS-3 preparations P14-A, N18-B, N18-A and N32-B fermented by post-weaning piglet faecal inoculum were air-dried on 13 mm filters with 10 µm pores (Merck Isopore™ membrane filter (Merck, Burlington, Massachusetts, USA)) and attached to sample holders containing carbon adhesive tabs (EMS, Washington, USA) and sputter coated with 12 nm tungsten (EM SCD 500, Leica, Vienna, Austria). The samples were analysed using Scanning Electron Microscopy (SEM) (Magellan 400, FEI, Eindhoven, The Netherlands) at the Wageningen Electron Microscopy Center. SEM images were recorded at an acceleration voltage of 2 kV and 13 pA and magnification of 10,000 and 25,000 times (Through Lens Detector). Unfortunately, we did not have fresh samples available for SEM analysis after *in vitro* fermentation of RS-3 preparations using pre-weaning piglet faecal microbiota.

2.6. Microbiota composition & data analysis

Microbiota composition was analysed in freeze-dried pellets obtained after *in vitro* fermentation, as described previously (Klostermann, Endika, Ten Cate, et al., 2023). In short, DNA was isolated, and duplicate PCRs were performed using purified DNA and the barcoded primer pair

515F (Parada, Needham, & Fuhrman, 2016)-806R (Apprill, McNally, Parsons, & Weber, 2015) to amplify the V4 region of the 16S rRNA gene. The PCR products were pooled in equimolar amounts, together with a no-template control and two mock communities of known composition, and the libraries were sent for Illumina Hiseq2500 (2 × 150 bp) sequencing (Novogene, Cambridge, UK). Raw sequence data of the 16S rRNA gene amplicons was processed using the NG-Tax 2.0 pipeline and default settings (Poncheewin et al., 2020). Taxonomy of each amplicon sequence variant (ASV) was assigned based on the SILVA database version 138.1 (Quast et al., 2013; Yilmaz et al., 2014). Data was analysed using R version 4.1.0 and the R packages phyloseq version 1.38.0 (McMurdie & Holmes, 2013), microbiome version 1.17.42 (Lahti & Shetty, 2012-2019) and microViz version 0.10.1 (Barnett, Arts, & Penders, 2021). Relative abundance of microbial taxa was calculated based on the 16S rRNA gene sequence read counts. Taxa that could not be identified at the genus level were renamed to the lowest classified taxonomic rank and sorted based on taxon abundance, e.g. the ASV of *Enterobacteriaceae* Family 01 is the most abundant ASV of an unidentified genus within the *Enterobacteriaceae*. Principle component analysis (PCA) and principle coordinate analysis (PCoA) were used to visualise the microbiota variation between substrates after centered-log-ratio (CLR) transformation of ASVs or Generalised UniFrac distances (i.e. taking phylogenetic relatedness and relative abundance of taxa into account, with an extra parameter α controlling the weight of abundant lineages (Chen et al., 2012)), and Unweighted UniFrac distances (i.e. taking phylogenetic relatedness between taxa, but not relative abundance of taxa into account), respectively. Sequences and sample information has been submitted to the European Nucleotide Archive of the European Bioinformatics Institute (EBI). The data can be found under the study accession number 'PRJEB65715'. ENA Browser (ebi.ac.uk)

3. Results

The *in vitro* fermentability of RS-3 preparations by pooled faecal inoculum obtained from pre-weaning (3-week-old, section 3.1) and post-weaning (7-week-old, section 3.2) piglets was studied. The RS-3 preparations differed in crystal type, Mw (DPn, number-based degree of polymerisation) and Mw distribution (PI, polydispersity index) (Table 1) and were prepared previously (Klostermann et al., 2021). The sample names have been recoded as compared to Klostermann et al. (2021) to improve intuitive understanding, with **P**: polydisperse; PI ≥ 1.3, **N**: narrow disperse; PI ≤ 1.25 - **DPn** and **crystal type**: A/B. *In vitro* digestion using porcine pancreatin and amyloglucosidase revealed that these RS-3 preparations differed in the proportion of rapidly digestible (RDS), slowly digestible (SDS) and resistant starch (RS) content (Klostermann et al., 2021) as defined by Englyst et al. (1992). P14-A and N18-B both contained a large proportion of *in vitro* digestible starch, whereas N18-A and N32-B were so-called intrinsic RS-3 (≥ 80 % RS). In the *in vitro* fermentation experiments also fully rapidly digestible soluble potato starch (SPS) was taken along as positive control.

3.1. Fermentability of RS-3 preparations by pre-weaning piglet faecal microbiota

RS-3 preparations P14-A, N18-A and N18-B (Table 1) and SPS were incubated with pooled faecal inoculum obtained from pre-weaning piglets for 48 h. Starch degradation, SCFAs, BCFAs, lactic and succinic acid were quantified over time, and changes in microbiota composition were evaluated.

The total starch degradation during *in vitro* fermentation of RS-3 preparations was evaluated (Fig. 1) by quantifying the remaining soluble and insoluble starch fraction at given time points (Table S2). The total starch recovery at all time points was normalised for starch recovery found at t0.

Soluble potato starch (SPS) (0 % RS) was fully degraded by pre-weaning piglet faecal microbiota within 24 h of incubation (Fig. 1).

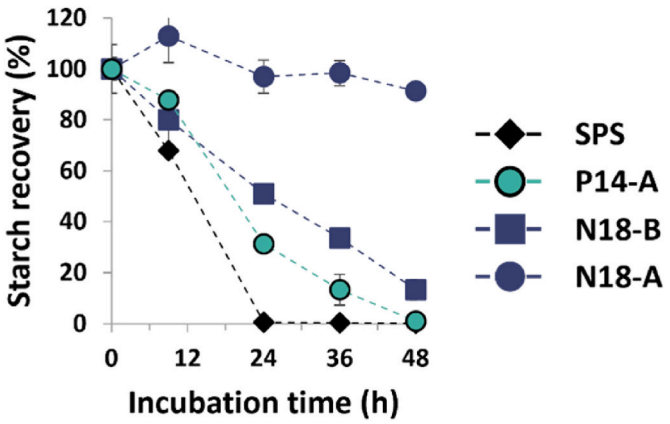

Fig. 1. Starch recovery after *in vitro* batch fermentation of RS-3 preparations P14-A, N18-B and N18-A and SPS with pooled pre-weaning piglet faecal inoculum for 0, 8, 24, 36 and 48 h. All starch recovery values were normalised for starch recovery found at t0. The average of biological duplicates is shown, the error bars represent the standard deviation and might be smaller than the marker used.

P14-A (polydisperse DPn 14 α-1,4 glucans in an A-type crystal (±15 % RS)) and N18-B (narrow disperse DPn 18 α-1,4 glucans in a B-type crystal (±26 % RS)) were degraded slower than SPS, with 30 % and 50 % starch remaining after 24 h, respectively. After 48 h of incubation, P14-A was fully degraded, whereas N18-B was degraded for ±85 %. Intrinsic RS-3 N18-A, prepared from similar Mw α-1,4 glucans compared to N18-B, but crystallised in an A-type crystal (±88 % RS), was hardly degraded during 48 h of incubation and thus behaved differently than the other RS-3 preparations in terms of *in vitro* fermentation by pre-weaning piglet faecal microbiota.

RS-3 preparations incubated without faecal inoculum (Table S3) showed that RS-3 preparations P14-A and N18-B partially solubilised during incubation (33.8 and 21.9 % at 24 h of incubation, respectively). However, after 24 h of incubation with faecal inoculum (Table S2), no soluble starch was detected anymore, indicating that soluble starch was fermented prior to insoluble starch.

After incubation of RS-3 preparations and SPS with pre-weaning piglet faecal microbiota, the SCFAs, BCFAs, lactic and succinic acid were quantified at different time points (Fig. 2).

Fermentation of SPS, P14-A and N18-B generated a similar total amount of acids after 48 h of incubation (on average 5.6, 5.8 and 5.9 μmol/mg substrate, respectively) (Fig. 2-A). The ratio between acetate, propionate and butyrate after fermentation differed, with SPS generating relatively more propionate, whereas P14-A and N18-B generated more butyrate (ratio 1 : 1.8: 2.1 (SPS), 1 : 2.1: 5.6 (P14-A) and 1 : 2.0: 4.1 (N18-B), after 48 h of incubation). Fermentation of P14-A also generated some succinate (0.3 μmol/mg substrate after 48 h), which was not observed after fermentation of N18-B. Intrinsic RS-3 N18-A generated a much lower total amount of acids (3.3 μmol/mg substrate after 48 h) compared to RS-3 preparations containing digestible starch (P14-A, N18-B) (Fig. 2). Nevertheless, fermentation of N18-A generated slightly more acids than medium blank (1.9 μmol/mg substrate after 48 h), indicating some additional fermentation, although no clear starch degradation was demonstrated (Fig. 1). The ratio of acetate, propionate and butyrate after 48 h of fermentation of N18-A was rather similar to that of the medium blank (N18-A: 1 : 1.1: 0.7, vs medium blank: 1 : 1.2: 0.6, after 48 h).

The results across treatments show that pre-weaning piglet faecal microbiota favoured to ferment the RS-3 preparations to relatively high amounts of propionate and butyrate, whereas low amounts of acetate were found. Although these low ratios of acetate are quite unusual for *in vitro* fermentation of dietary fibres, also previously higher levels of propionate than acetate were observed for some dietary fibres fermented

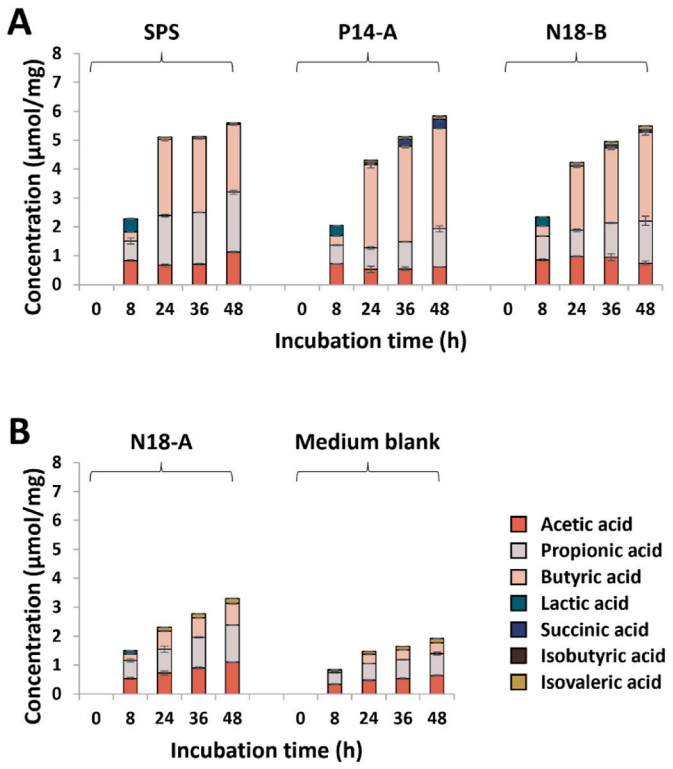

Fig. 2. SCFAs, BCFAs, lactic and succinic acid formation (µmol/mg substrate) during 48 h of incubation of RS-3 preparations with pooled pre-weaning piglet faecal inoculum. A: SPS and RS-3 preparations P14-A and N18-B, B: Intrinsic RS-3 N18-A and the medium blank. The average of biological duplicates is shown, the error bars represent the standard deviation.

with piglet faecal inoculum (Cao et al., 2023; Uerlings et al., 2020). Additionally, minor amounts of isovalerate (max 0.18 µmol/mg for N18-A) were generated, indicating slight proteolytic fermentation (Macfarlane, Gibson, Beatty, & Cummings, 1992).

Beta-diversity of microbial composition observed after 0, 24 and 48 h of *in vitro* fermentation of RS-3 preparations was determined and CLR-PCA, Unweighted UniFrac-PCoA and Generalised UniFrac-PCoA showed that the pooled faeces and the t0 digest clearly differed from the samples taken after 24 and 48 h of incubation (Fig. S1), indicating that *in vitro* fermentation selectively affected microbial communities, compared to the inoculum. Furthermore, the results show that SPS and RS-3 preparations containing a digestible fraction (P14-A and N18-B) differed from intrinsic RS-3 N18-A and the medium blank (Fig. S1).

The microbiota composition after 0, 24 and 48 h of *in vitro* fermentation of RS-3 preparations by pre-weaning piglet faecal microbiota were evaluated at family level (Fig. 3) and the microbiota composition of all samples at t0 appeared similar to the initial microbiota composition of the pooled faeces, and consisted for the largest part of approximately 14 % *Oscillospiraceae*, 13 % *Lactobacillaceae*, 10 % *Clostridiaceae*, 9 % *Bacteroidaceae*, 8 % *Lachnospiraceae* and 7 % *Christensenellaceae*, among others. Fermentation of SPS, P14-A and N18-B by pre-weaning piglet microbiota resulted primarily in an increase in relative abundance of *Streptococcaceae* (±45 %) (Fig. 3-A). Fermentation of SPS and P14-A also caused an increase in relative abundance of *Clostridiaceae* (±10–20 %) and *Veillonellaceae* (±20 %). Additionally, a slight increase in *Prevotellaceae* (±15 %) after 48 h of fermentation of P14-A was observed. Fermentation of N18-B by pre-weaning piglet faecal microbiota caused, next to the increase in relative abundance of *Streptococcaceae*, also an increase in *Prevotellaceae* (±30 %) after 48 h of incubation. Fermentation of N18-A by pre-weaning piglet microbiota increased the relative abundance of *Enterobacteriaceae*, *Prevotellaceae* and *Bacteroidaceae* (Fig. 3-B), but did not result in pronounced differences compared to incubation in the medium blank.

To have a deeper insight in the microbiota composition after fermentation, we analysed the microbiota composition at ASV level. The heatmap shows the relative abundance of ASVs present ≥2 % within a fermented sample and in total accounting for ±80 % of the relative abundance (Fig. S2). The relative abundance as shown at family level (Fig. 3) were primarily explained by only one ASV, with an increase of *Streptococcus* 01 after fermentation of SPS, P14-A and N18-B. All fermentations caused an increase of *Prevotella_9* 01 compared to the medium blank, except fermentation of SPS, lacking *Prevotella_9* 01 completely. For P14-A and P18-B, this increase in relative abundance of *Prevotella_9* 01 was primarily observed during the last 24 h of incubation. Fermentation of SPS and P14-A also caused an increase in relative abundance of *Megasphaera* 01 (*Veillonellaceae*), primarily during the last 24 h of incubation.

3.2. *Fermentability of RS-3 preparations by post-weaning piglet faecal microbiota*

The same RS-3 preparations were also incubated with pooled faecal inoculum obtained from post-weaning piglets, while one additional intrinsic RS-3 was included (N32-B; Table 1).

The total starch degradation was evaluated by quantifying the remaining soluble and insoluble starch fraction at given time points during incubation with (Fig. 4; Table S4) and without faecal inoculum (Table S5).

Complete degradation of SPS occurred during the first 24 h of incubation with post-weaning piglet faecal microbiota (Fig. 4), similar to results obtained for pre-weaning piglet faecal microbiota (Fig. 1). Approximately 50 % of P14-A and N18-B were degraded within the first 24 h of incubation, after which P14-A was fully degraded, whereas N18-B was degraded for ±80 % during the second 24 h of incubation (Fig. 4). P14-A and N18-B were degraded similarly by post- and pre-weaning piglet faecal microbiota (Fig. 4 vs Fig. 1), with a slightly higher degradation rate of P14-A compared to N18-B by both faecal inocula. In contrast, post-weaning piglet faecal microbiota degraded intrinsic RS-3 N18-A especially during the last 24 h of incubation for ±32 % (Fig. 4), whereas no clear degradation was observed by pre-weaning piglet faecal microbiota (Fig. 1). We also incubated another intrinsic RS-3 substrate, N32-B with a B-type crystal, using post-weaning piglet faecal microbiota, and also here we found clear degradation of the substrate for ±25 % during 48 h of incubation (Fig. 4). Although post-weaning faecal microbiota clearly degraded intrinsic RS-3, the degradation rate was much lower compared to the more digestible starches SPS, P14-A and N18-B.

The SCFAs, BCFAs, lactic and succinic acid were quantified for different time points (Fig. 5). Fermentation of SPS by post-weaning piglet faecal microbiota generated acetate, propionate and butyrate to similar levels after 24 and 48 h of incubation, (6.1 µmol/mg substrate) (Fig. 5-A), with a ratio of acetate, propionate and butyrate of 1 : 1.8: 1.5 after 48 h. Fermentation of P14-A and N18-B was more gradual with total acids of 4.5 and 6.1 µmol/mg substrate (P14-A) and 4.1 and 7.3 µmol/mg substrate (N18-B) after 24 and 48 h of incubation, respectively. Furthermore, fermentation of P14-A and N18-B differed in acetate, propionate and butyrate ratio (1 : 1.9: 1.3 for P14-A and 1 : 1.8: 0.5 for N18-B, at 48 h). N18-B thus generated more propionate, compared to SPS and P14-A. Fermentation of these substrates by pre-weaning piglet faecal microbiota led to the formation of similar total amounts of acids, but generally generated more butyrate.

Fermentation of intrinsic RS-3 substrates N18-A and N32-B generated lower amounts of acids (±3.5 and 3.3 µmol/mg substrate after 48 h, respectively) (Fig. 5-B), compared to partially digestible RS-3 substrates, in line with the starch recovery (Fig. 4). Nevertheless, these intrinsic RS-3 substrates generated slightly more acids compared to fermentation of the medium blank (2.0 µm/mg), with an acetate, propionate and butyrate ratio of 1 : 1: 0.5 (N18-A), 1 : 0.9: 0.5 (N32-B) and 1 : 0.5: 0.4

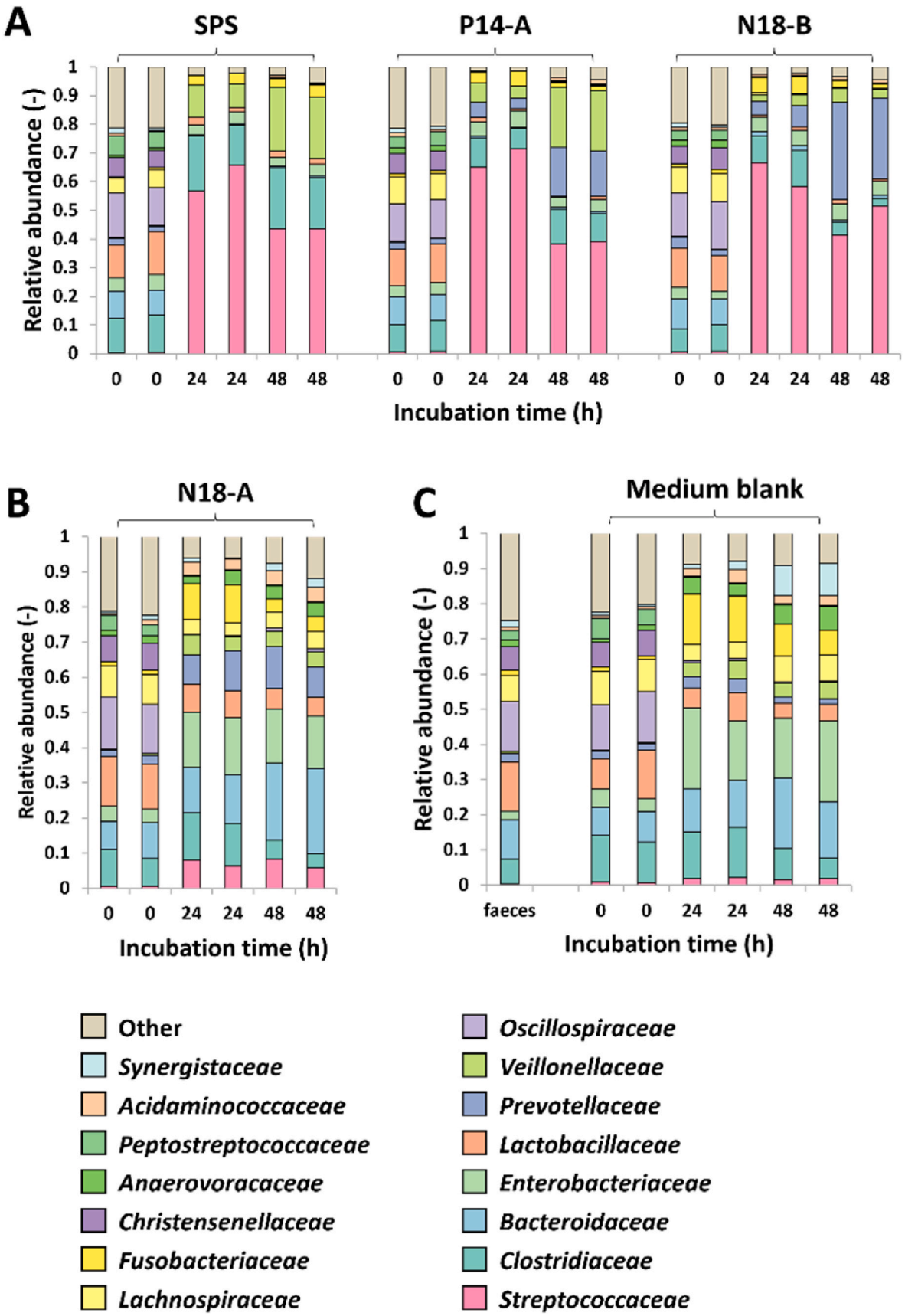

Fig. 3. Microbiota composition in relative abundance at family level during 48 h of duplicate incubations of RS-3 preparations with pooled pre-weaning piglet faecal microbiota. A: SPS and RS-3 preparations P14-A and N18-B. B: Intrinsic RS-3 N18-A. C: Medium blank and original pooled faeces. Results of both biological duplicates are indicated. The families shown are most abundantly present and contribute together to ≥80 % of the total relative abundance within a sample.

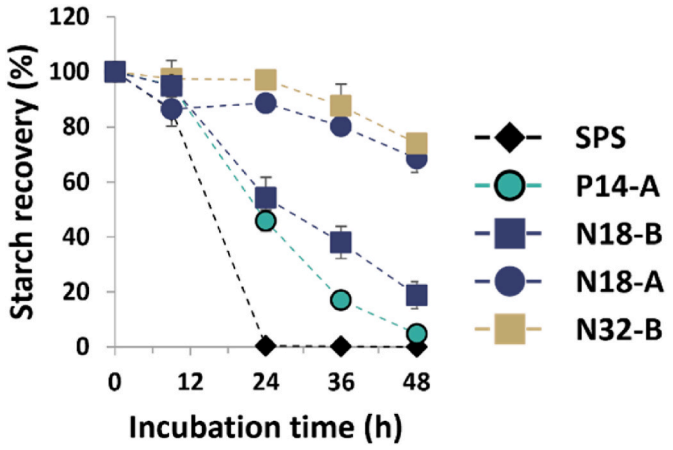

Fig. 4. Starch recovery during *in vitro* batch fermentation of RS-3 preparations and soluble starch with pooled post-weaning piglet faecal inoculum for 0, 8, 24, 36 and 48 h. All starch recovery values were normalised for starch recovery found at t0. The average of biological duplicates is shown, the error bars represent the standard deviation and might be smaller than the marker used.

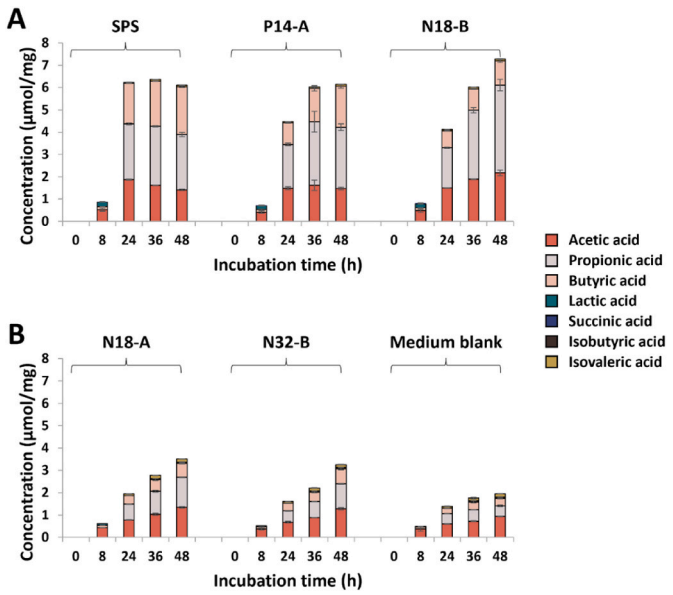

Fig. 5. SCFAs, BCFAs, lactic and succinic acid formation (µmol/mg substrate) during 48 h of incubation of RS-3 preparations with pooled post-weaning piglet faecal inoculum. A: RS-3 preparations containing >70 % digestible starch. B: Intrinsic RS-3 and the medium blank. The average of biological duplicates is shown, the error bars represent the standard deviation.

(medium blank) after 48 h of incubation. Comparing *in vitro* fermentation of intrinsic RS-3 N18-A by pre-vs post-weaning piglet faecal microbiota (Fig. 2-B vs Fig. 5-B), a similar total acid level (3.3 vs 3.5 µmol/mg substrate) with approximately similar ratios between SCFAs could be observed, indicating a rather similar microbial activity. Nevertheless, intrinsic RS-3 was clearly degraded more by post- compared to pre-weaning piglet faecal microbiota (Fig. 1 vs Fig. 4).

The microbiota composition obtained after 0, 24 and 48 h of *in vitro* fermentation of RS-3 preparations by post-weaning-piglet faecal microbiota was visualised using PCA of CLR-transformed relative abundance of ASVs and PCoA using Unweighted and Generalised UniFrac distances (Fig. S3) and indicated that the pooled faeces and digest t0 clearly differed from the samples taken after 24 and 48 h of incubation. Distinction between substrates based on β-diversity was much less clear for post-weaning piglet faecal microbiota, compared to pre-weaning piglet faecal microbiota (Fig. S1). Unweighted UniFrac-PCoA showed clear clustering of SPS, P14-A and N18-B both after 24 and 48 h of incubation (Figs. S3–B), indicating similar presence of unique taxa among these substrates at both time points. Interestingly, Unweighted UniFrac distances of fermented intrinsic RS-3 N18-A, N32-B and medium blank differed between 24 and 48 h, indicating different unique taxa among these substrates between 24 and 48 h of incubation.

The microbiota composition after 0, 24 and 48 h of *in vitro* fermentation of RS-3 preparations by post-weaning piglet faecal microbiota were evaluated at family level (Fig. 6) and the microbiota composition of all samples at t0 appeared similar to the initial microbiota composition of the pooled faeces and consisted of approximately 21 % *Clostridiaceae*, 18 % *Prevotellaceae*, 9 % *Lactobacillaceae*, 7 % *Lachnospiraceae*, 6 % *Ruminococcaceae* and 5 % *Oscillospiraceae*, among others. Fermentation of SPS caused an increase in relative abundance to ±30–40 % *Prevotellaceae*, ±10 % *Selenomonadaceae* and ±10–20 % *Veillonellaceae* (Fig. 6-A). Fermentation of P14-A and N18-B resulted in

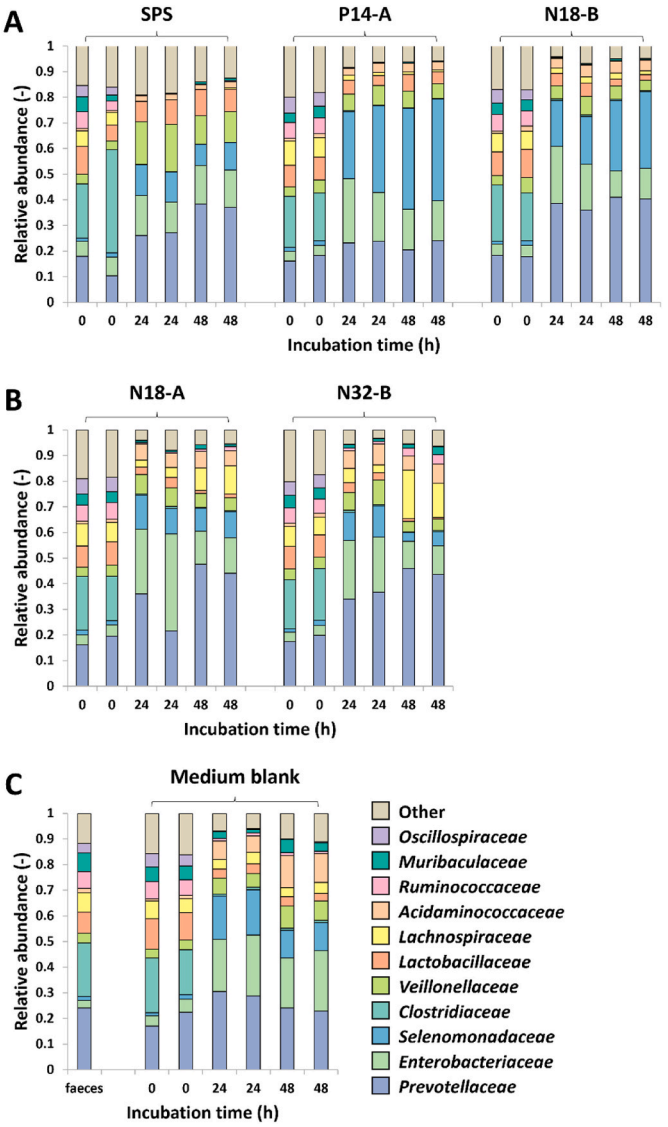

Fig. 6. Microbiota composition in relative abundance at family level during 48 h of duplicate incubations of RS-3 preparations with pooled post-weaning piglet faecal microbiota. A: RS-3 preparations containing >70 % digestible starch. B: Intrinsic RS-3. C: Initial pooled faeces and the medium blank. Results of both biological duplicates are indicated. The families shown are most abundantly present and contribute together to ≥80 % of the total relative abundance within a sample.

rather similar microbiota compositions with 40 % (P14-A) or ±20–30 % (N18-B) *Selenomonadaceae*, and ±20 % (P14-A) or 40 % (N18-B) *Prevotellaceae* after 48 h of incubation. Fermentation of intrinsic RS-3 N18-A and N32-B by pooled post-weaning piglet faecal microbiota resulted in a slightly different microbiota composition compared to the medium blank (Fig. 6-B, C). Especially after 48 h of incubation, an increase in relative abundance of *Prevotellaceae* to ±45 % (±23 % in medium blank) and *Lachnospiraceae* to ±10–15 % (±4 % in medium blank) was observed for intrinsic RS-3 N18-A and N32-B. Such pronounced increase in *Lachnospiraceae* was not observed after fermentation of starches containing a digestible starch fraction SPS, P14-A and N18-B (Fig. 6-A).

To further investigate the differences in microbiota composition after 24 and 48 h of incubation of RS-3 preparations with pooled post-weaning piglet faecal microbiota, we analysed the microbiota composition at ASV level, showing taxa contributing ≥2 % of the total relative abundance within a sample (Fig. S4). The increase in relative abundance of *Selenomonadaceae* as shown for fermented P14-A and N18-B (Fig. 6-A) was primarily explained by ASV *Selenomonas* 1. *Prevotellaceae* consisted of at least five different ASVs. *Prevotella_9* 01 was present in all fermented RS-3 substrates, whereas *Prevotella_7* 01 increased especially in relative abundance during the last 24 h of fermentation of SPS. Especially *Prevotella_9* 03 increased in relative abundance after 48 h of fermentation of intrinsic RS-3 N18-A and N32-B. Within the same timeframe also an increase in relative abundance of *Roseburia* 1 (*Lachnospiraceae*) was found for N18-A and N32-B.

3.3. Visual inspection of starch degradation by post-weaning piglet faecal microbiota

As we observed pronounced degradation of RS-3 preparations by post-weaning piglet faecal microbiota, we conducted SEM analysis to provide some preliminary insights in the degradation approach of insoluble starch by gut microbiota (Fig. 7).

The SEM image of the initial P14-A substrate shows that P14-A consisted of quite roughly clustered particles (Fig. 7-A1) of around 10–15 μm. After 24 h of incubation, the particles were covered by a biofilm (oval) with bacteria attached to the substrate (white arrows) (Fig. 7-A2, A3). The SEM image of the initial N18-B substrate shows that N18-B consisted of regularly shaped spheres of around 3 μm (Fig. 7-B1). After 24 h of incubation, holes appeared within those spheres (Fig. 7-B2, black arrow), which might indicate hydrolysis by extracellular amylolytic enzymes produced by the microbes. Additionally, bacterial cells were attached to the N18-B spheres (white arrow) (Fig. 7-B3). The SEM image of the initial N18-A substrate shows that N18-A consisted of elongated particles that were clustered together in spheres (Fig. 7-C1). After 48 h of incubation, these elongated crystals could be observed much better (Fig. 7-C2), probably due to degradation of an easier-accessible fraction. In addition, some bacterial cells were recognisable attached to the N18-A substrate (white arrow) (Fig. 7-C3). The SEM image of the initial N32-B substrate shows that N32-B consisted of quite regularly shaped spheres of around 3–7 μm (Fig. 7-D1). After 48 h of incubation some holes within the substrate appeared (black arrow). In addition, the surface of the N32-B spheres was rougher when compared to the initial substrate and some attached bacterial cells could be observed (white arrow) (Fig. 7-D2, D3).

4. Discussion

Weaning is a stressful period for piglets, often causing a disbalance in gut microbiota composition (Gresse et al., 2017), which might result in post-weaning diarrhoea. By *in vitro* fermentation of RS-3 preparations differing in crystal type, α-1,4 glucan chain length and RS content, we evaluated the prebiotic potential of such substrates for pre- and post-weaning piglets. Four RS-3 preparations (P14-A, N18-B, N18-A and N32-B) were incubated with inocula obtained by pooling faeces of pre- or post-weaning piglets and subsequently, starch degradation, organic acid production and microbiota composition were analysed. It should be noted that the pre-weaning piglets already had ad-libitum availability to creep-feed from 6 days of age, which could have influenced the microbiota composition of the faeces used as inoculum.

RS-3 preparations P14-A and N18-B contained a large digestible starch fraction (Table 1, (Klostermann et al., 2021)). Although the digestible starch is subjected to digestion in the small intestine, such substrates may partially escape digestion in pre-weaning piglets due to lower amylase activities in such piglets (Wiseman et al., 2000), rendering substrates available for large intestinal fermentation. In addition, Wang, Hu, Zijlstra, Zheng, and Ganzle (2019) found high relative abundance of starch-utilising enzymes being encoded within the metagenome of faecal microbiota of even up to four weeks post-weaning piglets consuming wheat starch and suggested that digestible starch is a major carbohydrate source for colonic microbiota (Wang et al., 2019). In contrast to digestible starches, which only conditionally reach the colon, starches that contain a high amount of RS (≥ 80 %) (Klostermann et al., 2021) can be expected to transit to the colon largely intact. However, such starches require microbes for degradation that express amylases with carbohydrate binding modules (CBMs), able to bind to the insoluble substrate (Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020). That such microbes should be targeted and specialised to degrade undigestible starches was demonstrated in our study where N18-A (±88 % RS) was hardly fermentable by pooled pre-weaning piglet gut microbiota, simply because the appropriate bacteria were not present at noticeable abundance. This was similar to a previous *in vitro* study showing that raw potato starch (RS-2) was poorly fermented by pre-weaning piglet faecal microbiota (Bauer et al., 2001). Indeed, it has been shown that among others, genes encoding starch degrading enzymes were lower in the bacterial metagenome of nursing compared to weaned piglets (Frese et al., 2015). Together this suggests that highly RS-enriched substrates might not be beneficial for pre-weaning piglets, simply because the specialised starch-degrading microbes are not (yet) present at high abundance. Nevertheless, we cannot exclude that intrinsic RS-3 would eventually be fermentable by pre-weaning piglet faecal microbiota using elongated fermentation time or repeated exposure simulating creep feed consumption prior to weaning, and thus, this requires further investigation. For pre-weaning piglets, more easily fermentable dietary fibres, such as GOS (Difilippo et al., 2016) or partially digestible starch might be more readily beneficial, let aside that such starch is not fully hydrolysed in time by pancreatic amylase in the upper GIT. Previously, it has been shown that waxy maize and normal maize starch, varying in amylose to amylopectin ratio, differently influenced the ileal and caecal gut microbiota composition of growing pigs (Luo, Yang, Wright, He, & Chen, 2015), indicating that such digestible starch is subject to fermentation, even in growing pigs.

So far, not many studies investigated the *in vitro* fermentability of dietary fibres using pre-weaning piglet faecal microbiota. Previously, it has been shown that wheat starch (containing digestible starch) was fermented slower by faecal microbes of 4-week-old pre-weaning piglets, compared to more readily fermentable dietary fibres inulin and lactulose (Awati, Bosch, Tagliapietra, Williams, & Verstegen, 2006). Here, we showed that pre-weaning piglet faecal microbiota fermented partially resistant starch slower than purely digestible starch, but still fermentation was almost complete within 48 h of incubation (Fig. 1). The observed increase in relative abundance in *Streptococcaceae* in our fermentation digesta (Fig. 3-A) was also found previously for cooked digestible wheat starch which was fermented using a faecal inoculum of growing pigs (Bui et al., 2020). In that same study, fermentation of granular wheat starch did not result in an increase in relative abundance of *Streptococcaceae* (Bui et al., 2020), suggesting that only easily fermentable starch was targeted by these bacteria. Similarly, a previous study in which different sources of (resistant) starch were compared on fermentability by pig faecal microbiota, showed that *Streptococcus* especially increased in relative abundance during fermentation of relatively accessible starches, especially at the initial stages of incubation

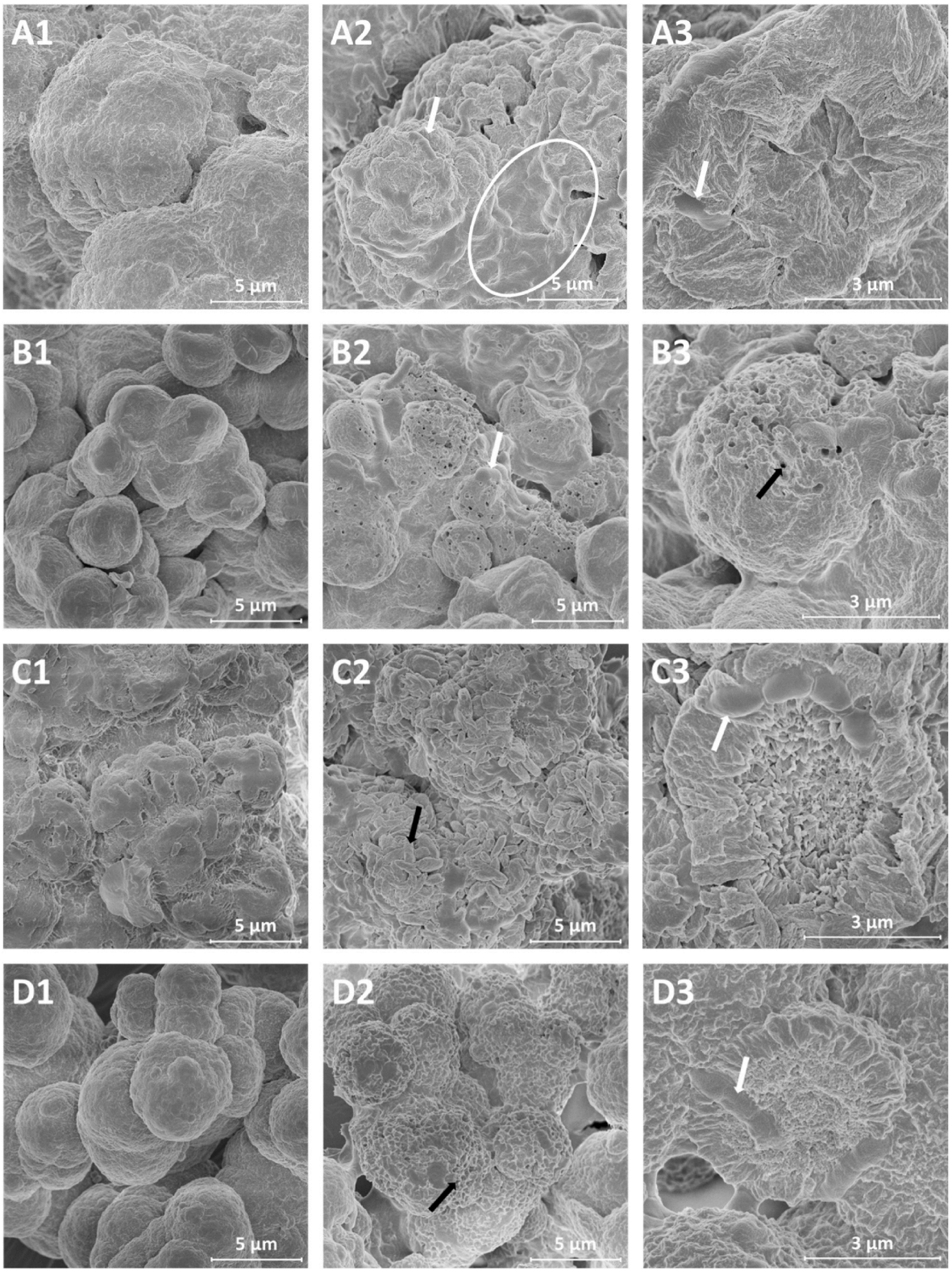

Fig. 7. Scanning Electron Microscope images of RS-3 preparations fermented by post-weaning piglet faecal microbiota. Figure A, B, C and D refer to P14-A, N18-B, N18-A and N32-B, respectively. Image 1 refers to the initial substrate, whereas image 2 and 3 refer to the substrate after 24 h of incubation (P14-A, N18-B) or 48 h of incubation (N18-A, N32-B). White arrows point towards bacteria, black arrows point towards specific starch structures, the white oval highlights the presence of biofilm.

(Warren et al., 2018). Indeed, some *Streptococcus* species are known to efficiently degrade soluble and raw maize starch and ferment it to lactate in mono-culture (Narita, Nakahara, Fukuda, & Kondo, 2004). *Streptococcus* could thus have contributed to the digestible starch degradation by pre-weaning piglet faecal microbiota in our study, producing lactate (present after 8 h of incubation, Fig. 2A), that could have been further metabolised to acetate, propionate and butyrate. Fermentation of partially digestible starch by pre-weaning piglet faecal microbiota in our study yielded low proportions of acetate and high proportions of propionate and primarily butyrate (Fig. 2A). Although starch is known to be butyrogenic, these proportions of butyrate are surprisingly high and even higher than previously shown for human faecal inoculum (Klostermann, Endika, Ten Cate, et al., 2023). The high amounts of butyrate produced could be explained by the increase in relative abundance of *Megasphaera* and to a lower extent *Clostridium sensu stricto* 1 (Fig. S2). Species within these genera are known to be significantly present in the piglet gut (Mahmud et al., 2023) and to produce butyrate. *Megasphaera* produces acetate, propionate and butyrate directly from lactate (Duncan, Louis, & Flint, 2004) and is additionally able to produce butyrate via the 4-aminobutyrate pathway (Anand, Kaur, & Mande, 2016), and could therefore have contributed to the SCFAs production and composition found. Fermentation of partially resistant P14-A and N18-B by pre-weaning piglet faecal microbiota also resulted in an increased relative abundance of *Prevotella*, primarily after 48 h of incubation. *Prevotella* harbours species with many carbohydrate degrading enzymes encoded on polysaccharide utilisation loci (PUL) (Accetto & Avgustin, 2015; Galvez et al., 2020). *Prevotella* species, such as *P. copri*, are able to produce succinate and acetate, but lack the ability to produce propionate (Franke & Deppenmeier, 2018). Since *Prevotella_9* 01 only increased in relative abundance after all soluble starch had been fermented by pre-weaning piglet faecal inoculum, it might be responsible for the degradation of the insoluble and resistant fraction of P14-A and N18-B preparations. Nevertheless, such abilities have not been described for *Prevotella* species before. In contrast, a recent study showed that specifically *P. copri* strains were only slightly growing on soluble starch, much worse than known starch degrading members of *Bacteroidetes* species *B. ovatus* and *B. thetaiotaomicron* (Fehlner-Peach et al., 2019). Partially resistant starches such as P14-A and N18-B are likely degradable by many different gut microbes, and therefore it is hard to predict the prebiotic potential of such substrates for pre-weaning piglets. Nevertheless, these partially resistant starches surely behaved like regular, beneficial dietary fibres for pre-weaning piglets, by promoting SCFAs production.

As indicated, and depending on the digestion kinetics, partially resistant starches such as P14-A and N18-B may be subject to ileal or colonic fermentation in post-weaning piglets. Both RS-3 preparations were fermented slower than purely digestible starch SPS by pre- and post-weaning piglet faecal inocula, both stimulating individually different microbial communities in pre-vs post-weaning faecal inocula. This indeed indicates that many different gut microbes can ferment substrates like P14-A and N18-B. It is worthwhile to note that N18-B was degraded less than P14-A, while causing a higher conversion to SCFAs after 48 h of incubation (Figs. 4 and 5A). This might be related to a difference in growth and interplay among the different microbes present (Fig. S4), resulting in a different efficiency in the production of the various metabolites.

Intrinsic RS-3 substrates N18-A and N32-B are different from partially resistant RS-3 preparations P14-A and N18-B, since they are >85 % undigestible by pancreatic α-amylase (Klostermann et al., 2021). The overall gut microbiome possesses more different α-amylases (Mukhopadhya et al., 2018; Ramsay, Scott, Martin, Rincon, & Flint, 2006), compared to the number of host α-amylases and therefore intrinsic RS-3 might be degradable by specific bacterial α-amylases, whereas it is not degradable by host α-amylases. Previously, we have shown that intrinsic RS-3 is much slower fermented than digestible starch (Klostermann, Endika, Buwalda, et al., 2023; Klostermann, Endika, Ten Cate, et al., 2023) and by distinct microbial communities.

Intrinsic RS-3 substrates N18-A and N32-B were slowly fermented by post-weaning piglet faecal microbiota, and starch degradation was especially observed between 24 and 48 h of incubation (Fig. 4), similar to what we found using human adult faecal inoculum (Klostermann, Endika, Ten Cate, et al., 2023). After 48 h of incubation, a pronounced increase in relative abundance of *Prevotella_9* 03 and *Roseburia* 1 to ±5–15 % was found, which were almost absent after 24 h. *Roseburia* 1 was fully absent after fermentation of all other starches and the medium blank, whereas *Prevotella_9* 03 was present at a relative abundance of 0–1 %. This indicates that intrinsic RS-3 specifically stimulated *Prevotella_9* 03 and *Roseburia* 1 using post-weaning piglet faecal inoculum. Other bacteria such as *Bifidobacterium adolescentis* and *Ruminococcus bromii* that harbour specialised RS-degrading machineries, are considered to be primary RS-degraders (Ze, Duncan, Louis, & Flint, 2012). However, we did not observe ASVs similar to *R. bromii* and *B. adolescentis*, indicating that also other gut microbes such as *Prevotella* and *Roseburia* species might be able to degrade intrinsic RS-3. This would be in agreement with an earlier statement, that *R. bromii* does not fulfil a key-stone degrading role in weaning piglets consuming wheat starch (Wang et al., 2019). These authors concluded that the metabolism of starch fermentation by pig faecal microbiota is dependent on cooperation between *Firmicutes* and *Bacteroidetes* (Wang et al., 2019), to which *Roseburia* and *Prevotella* belong, respectively.

Increased relative abundance of *Roseburia* was also shown when weaned piglets were fed 5 % raw potato starch (Yi et al., 2022). *Roseburia* produces butyrate from carbohydrates and is more often shown to increase in relative abundance during *in vitro* fermentation of RS (Wang et al., 2021). Some *Roseburia* species harbour a single α-amylase with multiple CBMs (CBM82 (Cockburn et al., 2018), CAZy database (Drula et al., 2022)) within the same protein (Ramsay et al., 2006), that could bind to insoluble starch. To the best of our knowledge, direct RS-degrading capabilities have not yet been shown for *Roseburia* species, although these bacteria are suggested to be secondary degraders of RS, that are likely able to bind and degrade partially pre-degraded RS (Cerqueira et al., 2020). *Prevotella* has a starch utilisation system-like (Sus-like) multiprotein complex as found for *B. thetaiotaomicron* (Accetto & Avgustin, 2015). However, *B. thetaiotaomicron* is unable to degrade RS on its own and relies on primary RS-degraders (Cerqueira et al., 2020) and additionally, *P. copri* was found to ferment soluble starch worse than *B. ovatus* and *B. thetaiotaomicron* (Fehlner-Peach et al., 2019). Nevertheless, it has been reported that some *Prevotella* strains harbour a CBM74-containing amylase (Valk, Lammerts van Bueren, van der Kaaij, & Dijkhuizen, 2016), together with a CBM26 domain being encoded in their genome (Photenhauer et al., 2022). Such complex present in *Prevotella* might be of importance to bind to insoluble starch as recently shown by Photenhauer and co-authors (2022) for keystone degrader *R. bromii* (Photenhauer et al., 2022). Since *Roseburia* 1 and *Prevotella_9* 03 ASVs specifically increased in relative abundance after incubation with intrinsic RS-3 and clear degradation of these substrates was shown using SEM (Fig. 7), it might be of interest to investigate *Prevotella* and *Roseburia* species from post-weaning piglet microbiota for their intrinsic RS-3 degrading capability.

5. Conclusions

We studied the fermentability of RS-3 preparations by pre- and post-weaning piglet faecal microbiota. Fermentation of RS-3 preparations by pre-weaning and post-weaning piglet faecal microbiota caused differences in microbial activity and composition. Both inocula fermented partially resistant RS-3 preparations quickly, whereas intrinsic RS-3 was only fermentable by post-weaning piglet faecal microbiota. Fermentation of RS-3 preparations by pre-weaning piglet faecal microbiota stimulated butyrate production and caused an increase in relative abundance of *Prevotella, Streptococcus* and *Megasphaera*. In contrast, post-weaning piglet microbiota fermented partially resistant RS-3

preparations to mostly propionate while stimulating *Prevotella* and *Selenomonas*. Intrinsic RS-3 fermentation by post-weaning piglet faecal microbiota resulted in an increase in relative abundance of specific phylotypes (ASVs) within *Prevotella* and *Roseburia*, which likely contributed to insoluble starch degradation and fermentation. Partially resistant RS-3 preparations may not have prebiotic potential for pre-weaning piglets, but might provide the common benefits of all dietary fibres by stimulating SCFAs production. Intrinsic RS-3 may have prebiotic potential in post-weaning piglets by stimulating more specific microbes, which requires further investigation.

CRediT authorship contribution statement

C.E. Klostermann: Conceptualization, Investigation, Methodology, Writing – original draft. **T.M.C. Quadens:** Investigation, Writing – review & editing. **L. Silva Lagos:** Conceptualization, Writing – review & editing. **P.L. Buwalda:** Conceptualization. **G. Bruggeman:** Resources, Writing – review & editing. **P. de Vos:** Conceptualization, Funding acquisition, Writing – review & editing. **J.H. Bitter:** Supervision, Writing – review & editing. **H. Smidt:** Writing – review & editing. **B.M. J. Martens:** Writing – review & editing. **H.A. Schols:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no financial or personal interest or belief that could affect our objectivity.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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