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In vitro fermentability of differently digested resistant starch preparations

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The *in vitro* fermentability of two resistant starch preparations type 2 (RS2) and type 3 (RS3) was investigated using human colonic microbiota. Prior to the fermentation experiments, samples were digested using two *in vitro* models, a batch (*ba*) and a dynamic (*dy*), as well as an *in vivo* method (*il*) for RS3. Digestion residues were fermented *in vitro* using a simple batch model lasting 24 h and a more sophisticated dynamic model enduring 72 h. During batch fermentation, metabolite productions and starch degradation rates were similar for RS2 and RS3 but higher for *dy*- compared to *ba*-digested samples. RS3*il* led to the lowest fermentability. Furthermore, increased butyrate ratios were observed for all preparations. The varying RS preparations behaved similarly in the dynamic fermentation but showed high SDs. Moreover, the fermentability was slow during the first 24 h, indicating that the microbiota needed an adaptation period to ferment RS. Propionate ratios increased at the expense of butyrate with exception for RS2*dy* showing an increase in acetate only. Differences in fermentability observed between the dynamic model, allowing a closer simulation of the *in vivo* behavior, and the batch model, recommended for screening purposes, could be due to the varying microbiota used.

Keywords: Batch *in vitro* fermentation / Dynamic *in vitro* fermentation / Resistant starch type 2 / Resistant starch type 3 / Short chain fatty acids

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1 Introduction

Resistant starch (RS), the portion of starch and starch products that resist digestion, can be classified into four general subtypes RS1–RS4 [1, 2]. RS1 includes physically inaccessible starch such as partly milled grains or seeds. RS2 refers to starch granules such as those found in green banana or raw potato. RS3 describes retrograded starch obtained through food processing or preparation and RS4 consists of chemically modified starches. In humans, RS consumption and subsequent fermentation is known to result in metabolic and physiological effects that may be related to disease prevention [3]. The majority of these effects are related to the fermentation in the large bowel. RS fermentation by the human colonic microbiota results in an increase in SCFA production, especially butyrate as observed in humans and also in rats in which human microbiota was introduced [4–7]. Butyrate is an important substrate for the oxidative energy metabolism as well as a signaling metabolite impacting on proliferation and differentiation of colonocytes [8, 9]. Furthermore, production of SCFA lowers the colonic pH which is thought to depress the conversion rate of primary to secondary bile acids and therewith lowers the carcinogenic potential of the colonic content [10]. Furthermore, RS is discussed as a potential prebiotic [3, 11, 12].

In humans, it is difficult to measure the fermentation of RS in the large bowel due to the inaccessibility of the proximal colon and portal vein. Therefore, alternative *in vitro* methods and models have been developed that mimic the events ongoing in the human colon. The *in vitro* models vary from simple, static batch culture systems to the more complex semicontinuous and continuous culture systems. The best model of choice depends on the aim of the study. Comparisons between investigations are hampered because of the wide range of incubation times, fecal inocula composition, substrate concentration, buffering of the medium, addition of protein and micronutrients, and analytical procedures. Even applying the same method in eight different labora-

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Abbreviations: BCFA, branched chain fatty acids; **RS,** resistant starch; **TS,** total starch

tories using different inocula led to remarkable differences in fermentability [13]. Any method chosen should simulate the *in vivo* condition as precisely as possible which is challenging to achieve because no reliable *in vivo* data exist. Studies with human volunteers mostly rely on breath hydrogen measurements or on the investigation of fecal samples. To enhance the reliability of the results, colonic samples rather than fecal samples should be investigated to follow the metabolite production in the human colon. Furthermore, it has to be kept in mind that *in vivo* studies with animals, inoculated either with defined bacterial species or a human fecal microbiota, cannot be compared directly with humans.

The substrates used for the *in vitro* fermentation experiment should either be *in vitro* or *in vivo* digested for a precise simulation of the following fermentation experiment. The digestion method has to be chosen carefully because it has an impact on fermentation as well [14]. In the present study, the fermentability of RS containing preparations was investigated using a batch and a dynamic *in vitro* fermentation model. The aim was to compare (i) two different types of RS and (ii) the effect of different predigestion, *i.e.*, two *in vitro* and an *in vivo* [15], on *in vitro* fermentability.

2 Materials and methods

2.1 Substrates

The RS3 containing carbohydrate source C*Actistar®, is a retrograded long chain maltodextrin product obtained after partial enzymatic hydrolysis and subsequent retrogradation of tapioca starch, produced according to United States Patent 6043229 [16] and obtained from Cargill (Vilvoorde, Belgium). The RS2 containing carbohydrate source is native high amylose maize starch and was obtained from Cerestar International (Neuilly-sur-Seine, France).

Prior to the *in vitro* fermentation experiments both samples were *in vitro* digested using a batch (*ba*) [17] and a dynamic (*dy*) [18] model resulting in the samples, RS3*ba*, RS3*dy*, RS2*ba*, and RS2*dy* as described and characterized by Fässler *et al.* [15]. Additionally, RS3 *in vivo* digested (RS3*il*) as described in [15] was used for the *in vitro* fermentation experiments.

2.2 Characterization of fermentation samples

Total starch (TS) contents in the samples used for the *in vitro* fermentation experiments were determined using the Megazyme TS assay kit (Megazyme) [19], with minor modifications as described in [15].

Protein content was determined as sum of amino acids [20]. RS3*il* was characterized for dietary fiber content by an enzymatic-gravimetric method [21] and for total nitrogen content according to Kieldahl at Cerestar–Cargill (Vilvoorde, Belgium). Nonprotein nitrogen (NPN) content was calculated by subtraction of protein from total nitrogen. Furthermore, the fat content was analyzed as sum of total fatty acids.

2.3 Batch *in vitro* fermentation experiment

One batch *in vitro* fermentation experiment was carried out using a mixture of fresh human fecal material from three healthy, nonmethanogenic humans under strictly anaerobic conditions as described by Lebet *et al*. [22]. Briefly, samples containing 100 mg of RS were hydrated overnight at 4°C in 8 mL of carbonate–phosphate buffer in 50 mL serum vessels. Two milliliters of inoculum containing fresh fecal material and buffer in a ratio 1:4 w/w was added to each vessel. The samples were incubated at 37°C in a shaking water bath and duplicates were taken after 0, 2, 4, 6, 8, and 24 h to follow the fermentation kinetics of selected parameters. Overpressure in fermentation vials was measured at 37°C through a canula with a manometer (MAT3-D20-B15, Angst & Pfister AG, Zurich, CH), to calculate the total gas volume at 20°C. A gas headspace sample was collected in a 60 mL syringe (Omnifix Luer Lock, B. Braun Melsungen AG, Melsungen, D) and 1 mL thereof was injected into a GC to measure the hydrogen concentration. Fermentation was stopped by the addition of 0.1 mL of a saturated $HgCl_2$ solution. The pH was measured, SCFA and residual dry matter were quantified by the methods described in detail by Lebet *et al*. [22]. The metabolites produced are given *per* 100 mg substrate fermented. Inoculum without any substrate served as blank. Lactulose (100 mg, Sigma, Buchs, CH), a well fermentable substrate, was used as positive control. Starch degradation during the *in vitro* fermentation experiment was determined by measuring the remaining TS in the freeze-dried fermentation residues.

2.4 Dynamic *in vitro* fermentation experiment (TIM-2)

Details of the TNO dynamic *in vitro* fermentation model have been described earlier by Minekus *et al.* [23] and Venema *et al.* [24]. The model simulates the proximal colon and consists of linked glass units with flexible walls inside. Peristaltic movements and body temperature are achieved by pumping water at 37°C into the space between the glass jacket and the flexible wall. The lumen of the model is equipped with hollow-fiber membranes (cut-off 50 kDa) to remove water and fermentation products, such as SCFAs. The strictly anaerobic system is kept at pH 5.8 by means of a pH sensor in combination with NaOH secretion. At the start of each experiment the model was inoculated with

30 mL of the standard microbiota (described below) and 20 mL ileal delivery medium [25] (containing digestible and indigestible carbohydrates as well as proteins). The microbiota was allowed to adapt to the conditions in the system for 16 h by introducing ileal delivery medium at a rate of 0.28 mL/h into the model. Then, the feeding was stopped for 1 h in order to allow the microbiota to ferment the available substrate. Thereafter the test feed (see below) was constantly introduced into the model at a rate of 2.3 mL/h for 72 h. For every substrate tested two experiments were carried out. Samples of the lumen and the dialysate were taken at 0, 24, 48, and 72 h. SCFA and ammonia were quantified as described previously by van Nuenen *et al.* [25] and are expressed as cumulative amounts produced during 72 h. Starch degradation during the *in vitro* fermentation experiment was determined by measuring the remaining TS in the freeze dried lumen samples.

2.4.1 Standard microbiota

The microbiota was prepared by mixing 100 g fresh feces obtained from five healthy volunteers with 140 g $NaCl_2$ solution (0.9% w/v), 40 g cysteine (BDH, Amsterdam, NL) and filled up to 900 mL with ileal delivery medium [25]. The mixture was incubated under strictly anaerobic conditions at 37°C for 4.75 h. Aliquots of 50 mL were frozen in liquid nitrogen and stored at −80°C until use. It has been shown before [24] that bacterial activities in the adapted inoculum, after keeping it at −80°C, was similar to those of the fresh mixture.

2.4.2 Test feed

Approximately 60 mL test feed was introduced into the model in 24 h. The test feed contains two-fold concentrated ileal delivery medium [25], except for the proteins which are concentrated 3.1-fold in the standard ileal delivery medium [25]. Carbohydrates were replaced by the test substrate containing 10 g starch. For the experiments with RS3*il* the amount of proteins added with the test feed was reduced to reach equal total protein contents in all the samples.

2.4.3 Statistical analyses

All the results are indicated as an average of duplicate fermentation samples ($n = 2$) obtained during *in vitro* fermentation.

3 Results

3.1 Composition of fermentation samples

All samples investigated were based on equivalent amounts of TS in the digestion residues (indigestible starch). Based on the different capacities of the batch and dynamic model, 100 mg and 10 g, respectively, of indigestible starch were introduced into the models *per* day. The 10 g of indigestible starch is within the average range entering the human colon [26]. Besides the indigestible starch, substrates contained nonstarch material ranging from 23 to 125/100 g as shown in Fig. 1. The nonstarch material present in *ba* and *dy* digested samples consists of additional enzymes and bile added during *in vitro* digestion. The protein content in *dy* digested samples was found to be higher (1.5/100 and 1.8/100 g substrates for RS3 and RS2, respectively) compared to *ba* digestion residues (0.6/100 and 1.2/100 g substrates for RS3 and RS2, respectively). Also glycine (component of bile) and other amino acids were found in higher amounts in *dy* compared to *ba* digestion residues [15]. RS3*il* contains 10.7/100 and 11.0/100 g fiber and protein, respectively. The ileostomy patients consumed, besides 40 g of RS3, a fiber-free diet, it is therefore likely that the main proportion of the fiber fraction in the effluent consists of pectins arriving from an orange juice drink given to the subjects [15]. In addition, substantial amounts of proteins and NPN, mainly from glucosamines (0.66/100 g), were found which probably originate from endogenous material. Fat was present in minor amounts only, 1.3/100 g. Approximately 30% in the *in vivo*, 25 and 20% in the *dy* and *ba* digestion residues could not be identified.

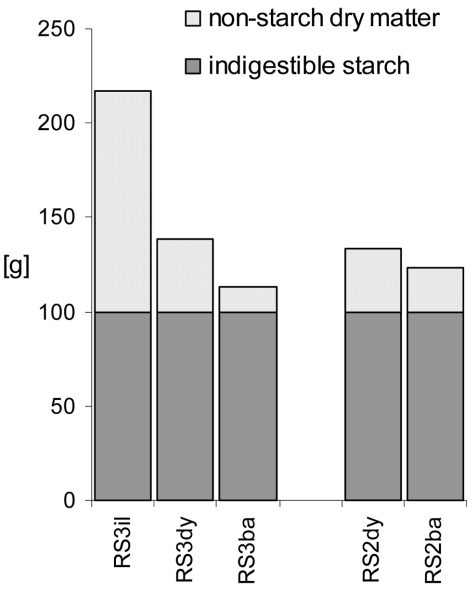

Figure 1. Composition of differently digested RS preparations used for the *in vitro* fermentation experiments. The samples are based on identical amounts of indigestible starch. For detailed composition of nonstarch fraction of RS3*il* see text.

3.2 Batch *in vitro* fermentation experiment

The control samples, lactulose and blank used for the batch *in vitro* fermentation experiment turned out to be very well

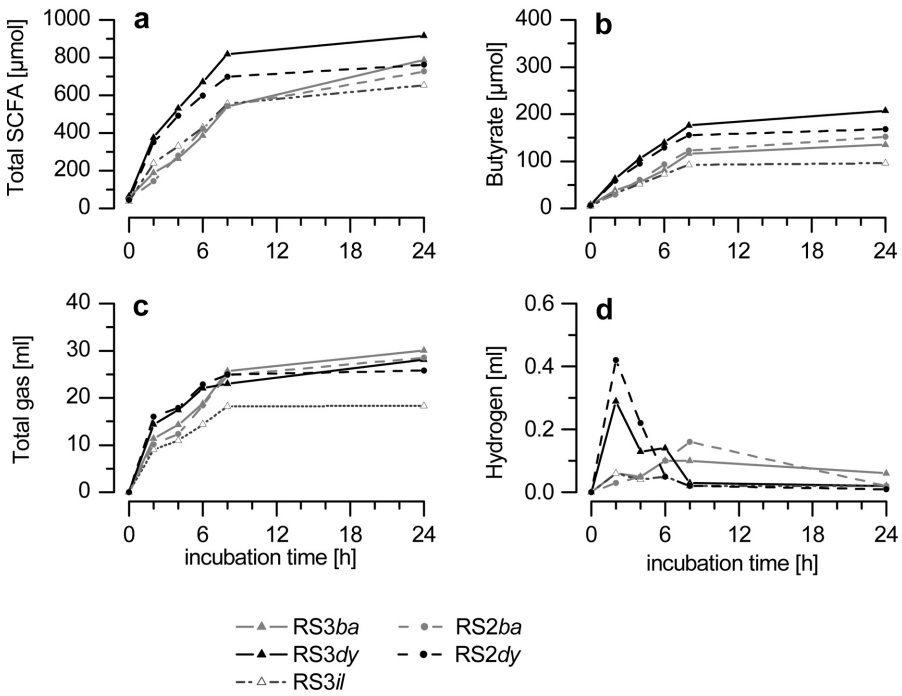

Figure 2. Average metabolite production during batch *in vitro* fermentation. (a) Total SCFA production, (b) butyrate production, (c) total gas profile, and (d) hydrogen accumulation. Results given *per* 100 mg total substrate fermented, $n = 2$.

Table 1. Production of total SCFA and butyrate (µmol/100 mg substrate fermented) and ratio of acetate/propionate/butyrate (ratio SCFA) (mol%) after 8 h batch *in vitro* fermentation

Time (h)	SCFA	RS3*ba*	RS3*dy*	RS3*il*	RS2*ba*	RS2*dy*	Blank[a)]
8	Total	541.9 ± 47.5	818.5 ± 100.2	555.4 ± 15.1	542.1 ± 54.3	697.6 ± 4.3	144.0
8	Butyrate	116.6 ± 5	176.1 ± 33.0	92.6 ± 4.1	123.0 ± 12.1	155.5 ± 2.0	26.4
8	Ratio	70:8:22	68:9:22	73:9:17	69:8:23	69:8:22	66:11:18

Mean values ± SD; $n = 2$.
a) $n = 1$.

within the average range of approximately 60 *in vitro* fermentation experiments (data not shown).

Total SCFA, butyrate, total gas, and hydrogen accumulation during batch *in vitro* fermentation experiment are shown in Fig. 2. SDs of gas and hydrogen accumulation were <11%. Total SCFA and butyrate productions resulted in SDs <10% whereas six values were found to be between 10 and 22%. *Dy* samples led to a higher metabolite production within the first phase of the *in vitro* fermentation experiment compared to *ba* samples. This is in agreement with the faster drop in pH from the *dy* samples compared to the *ba* samples (results not shown). Hydrogen production profiles show clear differences between samples. *Dy* digested samples caused a remarkable hydrogen accumulation at 2 h and dropped again thereafter. From *ba* digested samples a constant, slower hydrogen accumulation until 6–8 h was observed, whereas the accumulation from RS3*il* was very low at any time.

Total SCFA and butyrate productions and the ratios of acetate, propionate, and butyrate after 8 h batch *in vitro* fermentation are shown in Table 1; 8 h was chosen because starch was nearly completely fermented by this time. *Dy* digested samples led to the highest total SCFA and butyrate productions, *ba* and *il* digested samples were in a similar range with exception of the lower butyrate production for RS3*il*. The sum of isobutyrate and isovaleriate (branched chain fatty acids, BCFA) after fermentation of all samples was lower compared to the blank sample (not shown). This supports that BCFA was produced from fermentable protein present in the inoculum not from protein added with the sample. Interestingly, the higher protein content in RS3*il* did not influence BCFA production. All the samples

Table 2. Average starch degradation (mg/h) during the first 8 h of batch *in vitro* fermentation experiment

Time (h)	RS3*ba*	RS3*dy*	RS3*il*	RS2*ba*	RS2*dy*
0–2	5.7 ± 2.9	9.9 ± 1.2	4.6 ± 2.2	3.4 ± 1.0	11.6 ± 1.9[a)]
2–4	4.7 ± 0.7	10.6 ± 0.4	13.1 ± 2.0	6.8 ± 2.0	13.2 ± 4.7
4–6	14.2 ± 1.6	12.8 ± 0.3	15.9 ± 1.7	14.2 ± 2.4	12.2 ± 3.2
6–8	13.7 ± 0.8	7.2 ± 1.0	13.0 ± 1.6	11.8 ± 0.7	6.9 ± 0.5
8	22.9 ± 2.9	18.3 ± 0.5	6.2 ± 0.2	27.1 ± 2.6	11.3 ± 0.3
24	0.6 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	1.1 ± 0.0	0.5 ± 0.1

Residual starch content (mg) after 8 and 24 h of batch *in vitro* fermentation. Mean values ± SD; $n = 2$.
a) $n = 1$.

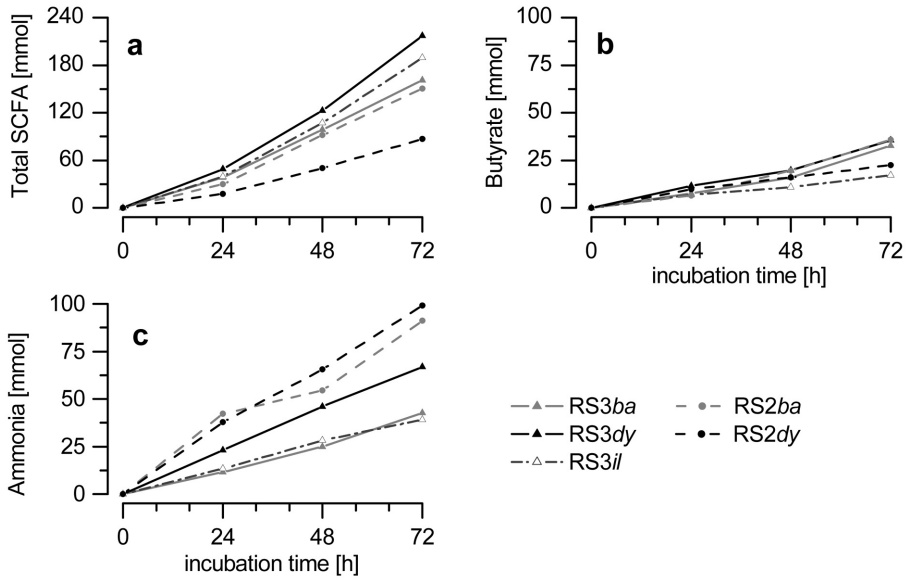

Figure 3. Average metabolite production during dynamic *in vitro* fermentation. (a) Total SCFA production, (b) butyrate production, and (c) ammonia production. Results given *per* total substrate fermented, $n = 2$.

resulted in high butyrate ratios after 8 h *in vitro* fermentation compared to the blank sample as well as to other dietary fiber sources fermented using the same model [27, 28].

Starch degradation rate during batch *in vitro* fermentation experiment can be followed in Table 2. *Dy* digestion residues led to a two-fold faster starch disappearance during the first 4 h of *in vitro* fermentation compared to *ba* digestion residues which is in agreement with the faster SCFA and gas production of *dy* samples compared to *ba* samples (Fig. 2). RS3*il* was degraded slowly from 0 to 2 h and faster from 2 to 4 h. Over 80% of the starch in *dy* digested samples was degraded at 8 h, which is higher compared to *ba* digested samples. However, it can be seen that the starch was completely fermented after 24 h in all the samples.

Overall, *in vitro* fermentation of RS3*il* and RS3*ba* showed similar amounts of total SCFA and butyrate accumulations until 8 and 6 h, respectively, thereafter metabolite production from RS3*il* slowed down. Gas production and hydrogen accumulation of RS3*il* was low compared to all the other samples.

3.3 Dynamic *in vitro* fermentation experiments

Figure 3 shows the average values of the cumulative total SCFA, butyrate, and ammonia production during dynamic *in vitro* fermentation experiments. SDs of total SCFA and butyrate production were <20% with exception of five values being between 20 and 28% and the butyrate production of *ba* digested samples at 48 and 72 h being between 33 and 54%. SD of ammonia production was <20% for all the samples with exception of RS3*il* which was higher. The constant feeding resulted in a steady increase in butyrate and ammonia (Figs. 3b and c), whereas total SCFA production was slightly slower during the first 24 h compared to

Table 3. Total production of SCFAs (mmol) and ratio of acetate, propionate, and butyrate (SCFA ratio) at 0 and after 72 h of dynamic *in vitro* fermentation experiment (mol%)

Time (h)	Metabolites	RS3*ba*	RS3*dy*	RS3*il*	RS2*ba*	RS2*dy*
0	Total SCFA	6.9 ± 3.9	11.7 ± 5.4	6.8 ± 2.5	7.2 ± 1.9	3.3 ± 2.7
72	Total SCFA	160.9 ± 15.4	217.3 ± 35.0	189.1 ± 32.6	150.2 ± 6.2	87.1 ± 1.4
72	Butyrate	32.6 ± 17.5	35.5 ± 1.8	17.0 ± 4.4	35.8 ± 19.0	22.5 ± 0.1
0	SCFA ratio	44:18:38	30: 7:62	49:13:38	36:17:47	3:61:35
72	SCFA ratio	31:48:20	35:49:16	34:57: 9	32:43:25	23:51:26
72	Total ammonia	42.7 ± 8.1	66.8 ± 2.6	39.2 ± 31.0	91.2 ± 5.3	99.1 ± 13.8

Butyrate and ammonia production after 72 h dynamic *in vitro* fermentation experiment (mmol). Results given *per* total substrate fermented. Mean values ± SD; $n = 2$.

Table 4. Average starch degradation (mg/h) during dynamically *in vitro* fermentation experiment

Time (h)	RS3*ba*	RS3*dy*	RS3*il*	RS2*ba*	RS2*dy*
0–24	0.24 ± 0.00	0.29 ± 0.06	0.28 ± 0.03	0.26 ± 0.01	0.37 ± 0.05
24–48	0.41 ± 0.06	0.42 ± 0.11	0.45 ± 0.02	0.43 ± 0.09	0.39 ± 0.01
48–72	0.49 ± 0.08	0.41 ± 0.00	0.49 ± 0.02	0.42 ± 0.06	0.45 ± 0.03

Mean values ± SD; $n = 2$.

the following 48 h for all samples. RS2*dy* led to clearly lower total SCFA productions compared to the other samples, which were in a similar range. Butyrate production was found to be comparable for all the samples until 48 h, thereafter the slope of RS3*il* and RS2*dy* slightly dropped. Cumulative ammonia production differed depending on the type of RS as well as the digestion method applied.

The cumulative total SCFA production and the ratios of acetate, propionate, and butyrate at time 0 and at 72 h as well as the total butyrate and ammonia produced after 72 h are shown in Table 3. The average total SCFA present at the beginning and after the *in vitro* fermentation experiment was found to be in a comparable range for all the samples, except for RS2*dy*, showing lower amounts. Similar butyrate accumulations after 72 h *in vitro* fermentation have been found for all the samples despite the high SDs for *ba* and *il* digested samples. Ratios of acetate did not change, propionate increased and butyrate decreased during the 72 h *in vitro* fermentation of RS with exception for RS2*dy*. Total ammonia produced after 72 h was found to be clearly higher after RS2 compared to RS3 fermentation. The ammonia production of RS3*il* led to an SD of 79%, it can therefore not be stated if the average value is closer to RS3*ba* or RS3*dy*.

Because 10 g starch/24 h is fed to the dynamic model, a degradation rate of approximately 0.42 g starch/h is expected assuming the starch is fermented at a constant rate. As can be seen in Table 4, during the first 24 h substrates were degraded at a clearly slower rate, with *ba* digested samples showing the lowest values. Less than 1 g starch originating from ileal delivery medium [25] was determined to be present in the models at 0 h; these low amounts could not have had an impact on the different starch degradation rates between the substrates from 0 to 24 h. From 24 to 72 h similar amounts of RS were degraded in all the samples investigated. The degradation rate of 0.42 g/h was exceeded in some cases due to the presence of unfermented starch previously introduced into the model.

Whether the fermentability of RS3*il* is closer to RS3*ba* or RS3*dy* cannot be concluded from the results obtained using the dynamic model because of the high SDs. In general, starch degradation of *dy* digested RS was faster during the first 24 h compared to *ba* digested RS. Ammonia productions after 72 h were higher for RS2 compared to RS3. Furthermore, RS2*dy* differed from the other samples by showing lower amounts of total SCFA and different SCFA ratios.

4 Discussion

4.1 Composition of fermentation substrates

The aim of the present study was to investigate and compare the fermentation of different types and structures of RS. Therefore, samples containing identical amounts of indigestible starch were fermented (Fig. 1). It cannot be concluded to what extent the additional nonstarch compounds present in the samples influenced fermentation

4.2 *In vitro* batch fermentation

Dy digested samples compared to *ba* digested samples have a faster metabolite production and starch degradation rate at the beginning of the *in vitro* fermentation experiment

(Fig. 2, Table 2). It was shown previously that *dy* digestion led to a lower crystallinity and lower amounts of analytically determined RS contents [15]. These differences may be responsible for the easier accessibility of *dy* samples for the microbiota at the beginning of the *in vitro* fermentation experiment. Moreover, it is hypothesized that the additional nonstarch substrates in *dy* digestion residues led to a higher microbiota activity resulting in a faster starch degradation as well.

Dy digested samples led to higher amounts of SCFA produced during batch *in vitro* fermentation compared to *ba* digested samples. This supports again the hypothesis that different starch structures obtained by different digestion methods had a higher impact on fermentation than the types of RS. This is in agreement with the results of an interlaboratory study [13], where it was concluded that differences in structure were responsible for different fermentation patterns for two RS3 and an RS2 preparation.

The amounts of starch degraded after 8 h in the batch *in vitro* fermentation experiment differed depending on the sample investigated. However, degradation after 24 h was completed in all samples (Table 2). Edwards *et al*. [13] found considerable interindividual differences in starch degradation after 24 h batch *in vitro* fermentation using fecal samples of 36 different donors. They showed SDs of 50 and 98% for the degradation of RS2 from raw potato starch and RS3 from retrograded potato starch. They concluded that these variations reflect a true *in vivo* RS fermentability. However, Edwards *et al*. [13] did not carry out a digestion pretreatment prior to the fermentation experiment. Additionally, their model, their RS preparations, and their inocula differed compared to the present study which makes direct comparisons difficult. In general, it can be assumed that for batch *in vitro* fermentation experiments *dy* and *ba* digested RS preparations led to differences, whereas the type of RS only had a minor impact on fermentation. Furthermore, it cannot be predicted whether the fermentation patterns of *ba* or *dy* digested starch are closer to the ones of *in vivo* digested starch. Although RS3*il* best reflects the physiological conditions it has to be mentioned that the high amounts of nonstarch material present in RS3*il* influenced the fermentation pattern.

4.3 Dynamic *in vitro* fermentation

All samples led to a slower SCFA production during the first 24 h compared to the following 48 h of dynamic *in vitro* fermentation experiment (Fig. 3) which correlates with the slower starch degradation, except for RS2*dy* (Table 4). The slower fermentation could be due to a lower enzyme activity during the first 24 h because the adaptation period of 16 h was not long enough. Alternatively it could be that the microbiota preferred fermentable compounds added with the feed during the 16 h adaptation period resulting in a slower adaptation to RS as a substrate. The starch added with the ileal delivery medium [25] was found to be practically gone (<1 g remaining) at the start of the *in vitro* fermentation experiment. However, during this time also 0.4 g xylan and 0.4 g arabinogalactan were introduced into the model which are known to be poorly fermented; for arabinogalactan this was shown recently using the batch *in vitro* fermentation model [29]. Furthermore, it is possible that some of the proteins added with the ileal delivery medium remained in the model at the beginning of the experiment. It is therefore likely that at the beginning of the experiment some fermentable substrates were still present in the lumen and may have influenced the fermentation patterns.

The average production of total SCFA after the adaptation period, at the start of the experiment was found to be in a comparable range for all the samples with exception of RS2*dy.* Whether the lower amount of SCFA and different SCFA profile of RS2*dy* was influenced by the uncharacteristic SCFA production during the adaptation period remains speculative. However, SDs for all the samples were high, although the microbiota was taken from the same batch and the feed was prepared identically for all the experiments. Duplicate fermentation analyses were carried out at the same day using two models but identical microbiota aliquots. This indicates that the SDs obtained between duplicate experiments are most likely due to slightly different conditions in the models. The low amounts of total SCFA and acetate in RS2*dy* at 0 h were observed using both models indicating that during both experiments the microbiota must have followed different pathways compared to the experiments with the other substrates.

The average ratios (excluding the values for RS2*dy*) after the 16 h adaptation period for acetate, propionate, and butyrate are 40 ± 8, 14 ± 5, and 46 ± 11, respectively. Studies done previously, using the same model and ileal delivery medium but different inocula, resulted in ratios of 69 ± 8:8 ± 4:23 ± 8 after 48 h [30] and 46:19:36 (SD<10%) [25] after 96 h. These results are in a similar range as the average values found in the present study, however, the SDs in this study are higher. Whether the SDs were influenced by the 16 h adaptation period, which may have been too short to obtain a similar and stable flora for all the experiments, cannot be concluded.

Although RS2*dy* led to lower amounts of SCFA during the first 24 h compared to the other samples (Fig. 3) starch degradation was found to be high (Table 4). This was not expected, because carbohydrate degradation and SCFA production are generally inter-related [31]. Therefore, it may be assumed that higher amounts of other, not measured metabolites were produced, for example CO_2.

Dynamic *in vitro* fermentation of 72 h did not ferment the RS completely. Due to the continuous feeding, a little amount of starch enters the model at $t = 72$ h. Furthermore, the inoculum used was prepared differently compared to other *in vitro* studies making comparison difficult. However, *in vivo* studies have shown that considerable individual differences in RS metabolism exist depending on the amount of starch given and the microbiota used [4, 32]. Hylla *et al.* [33] found large interindividual variations in RS degradation in an *in vivo* study feeding 55 g RS2 (same substrate as used in present study) *per* day, whereas van Munster *et al.* [6] found an almost complete absence of residual starch in the feces after adding 45 g/day of the same RS2 preparation to a standardized diet. Whether the starch would have been degraded to the same extent using different donors and a fresh inoculum without any incubation steps cannot be predicted.

The lower amounts of ammonia produced by the microbiota in the presence of RS3 compared to RS2 are in correlation with the results obtained by Heijnen *et al.* [34] feeding healthy men the same RS2 preparation as used in present study and an RS3 preparation from maize starch. Protein fermentation products including ammonia, are believed to be carcinogenic, this indicates that RS3 might act preventive against colorectal cancer [35].

As a consequence of the high SDs obtained using the dynamic *in vitro* fermentation model, no differences in SCFA productions between either RS preparation or varying digestion methods can be drawn. Furthermore, as already seen for batch fermentation it cannot be concluded whether the fermentation patterns of RS3*ba* or RS3*dy* are closer to RS3*il*. It can be inferred from the data that the ammonia accumulation was higher during RS2 compared to RS3 fermentation. Overall these results indicate that the two RS containing samples may be fermented similarly in the human colon.

4.4 Comparison of *in vitro* fermentation models

It is difficult to compare fermentation patterns obtained by the different models. Minor differences in study designs and experimental parameters may influence metabolite production significantly. In the present study, two models differing in a large number of aspects were used. The main goal of the batch *in vitro* fermentation model is to investigate the rate of substrate degradation and the amounts of metabolites produced when feeding the microbiota a defined amount of substrate. With the dynamic model, the events in the proximal colon are simulated by trying to maintain a constant microbiota milieu by adding substrate and removing metabolites continuously. In contrast to earlier work, this was the first study to ferment identical RS preparations in both models. Therefore, it seems interesting to discuss some parallels and discrepancies when interpreting the *in vitro* fermentation of RS using different models.

The ratios of SCFA at the start of the dynamic *in vitro* fermentation and of the blank sample after 8 h batch *in vitro* fermentation are presented in Tables 1 and 3. Measurements in sudden death victims showed ratios for acetate, propionate, and butyrate of 57:21:22 in the cecum, and 57:22:20 in the rectum [36]. These results are closer to those obtained with the blank sample from batch fermentation. However, considerable variations in SCFA ratios in human feces have been shown previously [37]. The different conditions in the models at the beginning of the experiment might have influenced subsequent RS fermentation in varying ways.

Butyrate ratios after RS fermentation are expected to be higher compared to other complex carbohydrates [38]. High butyrate ratios at the end of the fermentation experiment were observed using both models (Tables 1 and 3). However, during dynamic *in vitro* fermentation butyrate proportions decreased over time.

In contrast to the other samples, *dy* digested starch degraded more easily from the very beginning in both *in vitro* fermentation experiments. This is interesting, considering that the kinetics of substrate degradation in the two fermentation models cannot be compared directly because, in the batch model, all the substrate is given at once whereas during dynamic fermentation RS is fed continuously along with other food compounds.

It can be concluded that the results obtained with the simple batch model were more reproducible, whereas higher variations between experiments were observed using the more sophisticated, dynamic model. Both models have unique applications in the field. The dynamic model simulates the behavior of the human colon *in vivo* more precisely whereas the batch model is better for screening of compounds. Depending on the aim of the study, the batch or the dynamic model can be more suitable. When choosing a model it is important to be aware of its limitations and to define all the parameters very well before carrying out the experiment.

To simulate the events in the human colon it is important to use substrates as they enter the colon. Therefore, using substrates as eaten and carrying out an *in vitro* or *in vivo* digestion method prior to the fermentation experiment is imperative. Nevertheless, it has to be taken into account that the digestion method will influence the subsequent fermentation experiment as well. Accordingly, it seems inappropriate to make comparative statements about fermentation characteristics of substrates in all the cases that were not tested in the same experiment with the same microbiota and methodology.

To be able to compare more substrates, standardization of both the batch and the continuous methods would be imperative. However, whether this would help to obtain more consistent results remains to be elucidated.

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