Breakdown of Amylomaize Starch Granules in Gnotobiotic Rats Associated with Four Bacterial Strains Isolated from Conventional Rat Microflora

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Abstract: Balance studies in germ-free (GF) and conventional (CV) rats have shown that in-vivo breakdown of amylomaize starch depends on endogenous and bacterial enzymes. In GF rats, one-third of amylomaize starch is resistant to endogeneous digestion, whereas in CV rats starch is totally degraded. In order to study the contribution of endogenous and bacterial enzymes, the in-vivo breakdown of amylomaize starch was compared in GF and in gnotobiotic rats associated with bacterial strains isolated from the conventional rat microflora. These bacteria were (i) a non-amylolytic *Peptostreptococcus* (GNP rats), (ii) an amylolytic strain of *Eubacterium* able to hydrolyse resistant starch (GNE rats), and (iii) an association of *Eubacterium* with three other bacterial strains (*Veillonella*, *Enterococcus* and *Peptostreptococcus*) able to ferment the products of starch hydrolysis (GNT rats). Various studies were performed to assess the starch breakdown in the caecal contents: balance studies, light and electron microscopy and analyses of bacterial metabolites. The results show that in GNP as in GF rats, around 65% of amylomaize starch was resistant to digestion. Digestive utilization of starch was significantly improved in GNE rats (from 68 to 92%) and was further enhanced to 97% in GNT rats. The increase in starch degradation in the caecum of rats was related to a higher amylase activity and led to an increase in production of bacterial metabolites, and to a change in the proportions of short-chain fatty acids, lactic acid and succinic acid. Ultrastructure study of the starch in the caecal contents showed a progressive granule hydrolysis in the order GF, GNE and GNT rats: (i) an endocorrosion of the central part of the starch granules in GF rats, (ii) a peripheral digestion with bacteria penetrating the granules in GNE rats, and (iii) a more pronounced hydrolysis in the caecum of GNT rats where the outer part of the granules were almost entirely hydrolysed. These results suggest that starch breakdown in the hindgut is not only due to amylolytic bacteria but also to the contribution of other glycolytic strains able to utilize the products of starch hydrolysis.

Key words: resistant starch, digestive utilization, electron microscopy, fermentation metabolites.

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INTRODUCTION

Resistant starches occur naturally in uncooked starchy diets or are produced during food processing (Englyst and Macfarlane 1986). They are known for their low susceptibility to pancreatic α -amylolysis and availability for fermentation by the microflora in the large gut (Macfarlane and Englyst 1986). The uncooked amylo-maize starch presents an X-ray diffraction B-type (Zobel 1988) and shows a very resistant peripheral shell which remains as residual ghost after in-vitro amylolysis (Gallant *et al* 1973, 1992; Gallant and Bouchet 1986).

Previous in-vivo studies have shown that native amylo-maize starch is only partly digested by germ-free rats (Andrieux and Sacquet 1986; Andrieux *et al* 1989); in the caecal content, starch granules showed an endo-corrosion (Andrieux *et al* 1992) similar to that observed, *in vitro*, during amylolysis by pig pancreatic α -amylases (Gallant 1974). In conventional as in germ-free rats, only 60% of the amylo-maize starch was digested in the small intestine (Andrieux *et al* 1992) and the residual starch, not yet digested by the endogenous enzymes, was totally degraded. Starch degradation is accompanied by caecal microflora fermentation, producing short-chain fatty acids and reducing bacterial activities implicated in toxicological functions (Mallett *et al* 1988; Andrieux *et al* 1989). From the conventional rat microflora and using a medium containing autoclaved amylo-maize starch as substrate, we isolated amylolytic strains from *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Eubacterium* and *Lactobacillus* genera, which partially degraded amylo-maize starch *in vitro*, but were unable to degrade the resistant fraction of this starch *in vivo* when they were inoculated into germ-free rats (Pacheco Delahaye 1990). In the present study, using as substrate the resistant fraction of amylo-maize starch from the caecal content of germ-free rat, we isolated four glycolytic strains able to degrade more than 90% of this resistant starch when they were incubated together *in vitro*. With regard to the action of endogenous and bacterial enzymes, we compared the in-vivo amylo-maize starch breakdown in the caecum of germ-free rats and of gnotobiotic rats associated with the amylolytic anaerobic bacteria, *Eubacterium* sp B86 itself able to hydrolyse amylo-maize starch (Andrieux *et al* 1990), with a strain of *Peptostreptococcus* which did not hydrolyse starch or maltose, or with the association of *Eubacterium*, *Peptostreptococcus*, *Veillonella ratti* able to hydrolyse maltose and maltodextrins, and *Enterococcus faecalis* able to hydrolyse maltose (Pacheco Delahaye 1990). Three different studies were investigated in gnotobiotic rats and compared with germ-free rats (i) balance studies which measured the amount of resistant starch; (ii) light and electron microscopy which assessed the behaviour of starch susceptibility during hydrolysis; and (iii) analyses of bacterial metabolites by which the end-products of bacterial metabolism were determined.

MATERIALS AND METHODS

Selection and identification of the bacterial strains

Agar and liquid 8H culture medium, pH 7.8, contained (g litre⁻¹): bacterio-peptone, 5; bacterio-tryptone, 10; liver extract, 5; and resistant starch, 5. This resistant starch, derived from the caecal contents of germ-free rats fed amylo-maize starch diet, was washed three times with sterile NaCl (9 g litre⁻¹) and added under sterile condition to the culture medium. Anaerobic bacteria were selected from plates containing the resistant starch medium, inoculated with 10⁻⁸ and 10⁻⁹ dilutions of faeces of conventional rats fed amylo-maize starch, and incubated for 72 h in an anaerobic chamber. In the plates cultures, from 108 colonies, 18 starch-hydrolysing strains were identified by the clear zones generated around the colonies. In liquid medium, 30% of the resistant starch was only degraded by these amylolytic strains when they were inoculated alone. The association of these amylolytic strains with one or several bacterial strains which did not produce clear zones, led to an increase of resistant starch degradation up to 50%. Among 60 mixed cultures, the most efficient combination degraded more than 90% of the resistant starch *in vitro*. The bacteria were identified as amylolytic *Eubacterium* sp B86, *Veillonella ratti* (able to hydrolyse maltose and maltodextrins), *Enterococcus faecalis* (able to hydrolyse maltose) and *Peptostreptococcus* (unable to hydrolyse starch nor maltose). These bacteria were characterized on the basis of the Gram reaction, the morphology, the ability to ferment carbohydrates and the profile of fermentation products (Pacheco Delahaye 1990).

In-vivo experiments

Four groups of six adult male germ-free (GF) Fisher 344 rats, body weight 360 $\pm$ 18 g, were used. They were maintained in Trexler-type isolators, either in GF state or in gnotobiotic state (GN), associated with the non-amylolytic *Peptostreptococcus* sp 610 (gnotobiotic rats GNP), the amylolytic *Eubacterium* sp B86 alone (gnotobiotic rats, GNE), or *Eubacterium* sp B86 in association with *Peptostreptococcus* sp 610, *Enterococcus faecalis* sp 84 and *Veillonella ratti* sp 36 (gnotobiotic rats, GNT).

Rats were fed *ad libitum* a semisynthetic diet (Andrieux and Sacquet 1986), sterilised by γ -irradiation in double vacuum-sealed bags and containing 580 g litre⁻¹ amylo-maize starch (Eurylon VII, Roquette, France).

Inoculation of the strains occurred 2 weeks after the GF rats received amylo-maize starch diet. *Eubacterium* implantation need direct injection, into the caecum of anaesthetized rats, of 1 ml of the fresh culture (liquid

8H medium containing resistant starch). Fresh cultures of the other strains were obtained in brain heart infusion (BHI) medium and inoculated by stomach probe. The implantation of strains was controlled by enumeration of the bacteria in the faeces of gnotobiotic rats. Faeces were diluted in anaerobic chamber and dilutions from 10^{-6} to 10^{-9} were inoculated in plates containing either 8H medium containing resistant starch or BHI medium. From faeces of GNT rats, *Eubacterium* was identified in resistant starch medium by clear zones around the colonies; the other strains were differentiated by the morphology of the colonies and microscopic study.

Each group was fed for one month on the diet then a starch balance study of 3 days was done. Rats were then killed at 09.00 h, in a fed state as shown by presence of food in the stomach. One part of the caecum content was immediately frozen until required for analysis of starch and fermentation metabolites; another part was immediately treated for light microscopy, for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) for ultrastructural analysis of starch.

Starch analysis

In diet and faeces, starch was dispersed in dimethylsulphoxide ($800 \text{ ml litre}^{-1}$) by autoclaving for 20 min. Samples of this suspension, containing at most $5 \text{ g starch litre}^{-1}$, were then hydrolysed at 55°C for 3 h in a 10 mM phosphate buffer (pH 4.5) by amyloglucosidase (EC 3.2.1.3, Optidex 1200, Roquette frères, Lestrem, France). Glucose was determined enzymatically in samples with glucose oxidase (EC 1.1.3.4, Biotrol, Paris) before and after starch hydrolysis. The total starch content were corrected for free glucose in the faeces. Analyses were performed in triplicate.

Amylase activity

This was determined using an enzymic colorimetric test with a chromophore blue as substrate (Phadebas, Pharmacia, France). Activity was expressed as U g^{-1} fresh weight, one unit of amylase being equivalent to $1 \mu\text{mol}$ maltose produced per min at 37°C .

Fermentation metabolites

Short-chain fatty acids (SCFA) were analysed using gas chromatography after water extraction. The chromatograph was a GC 121 Del (Delsi) apparatus with a flame-ionisation detector and a semi-capillary column ($15 \text{ m} \times 0.53 \text{ mm}$) impregnated with SP 1000. Isobutyric acid was used as internal standard. The integrator was a Delsi Enica 21 apparatus.

L-Lactic and D-lactic acids and succinic acid were determined enzymically (Boehringer-Mannheim).

Statistical analysis

Results are expressed as means with their standard errors. Values were compared by analysis of variance and the Newman-Keuls test using STAT ITCF software.

Microscopic studies

Light microscopy

A part of the caecal contents, unwashed, was embedded in 30 g litre^{-1} agar in water at temperature below 50°C . When the agar had solidified, small cubes of sample were cut and fixed for 1.5 h at 4°C in 30 g litre^{-1} glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, then washed several times in buffer followed by deionized water. Dehydration was done in an ethanol gradient, then propylene oxide before embedding in Epon resin. Polymerization was performed in an oven 40°C overnight, then 60°C for 4 days. Sections of $2 \mu\text{m}$ thickness were stained using fast green (10 g litre^{-1}) and iodine solution (0.02 M). Sections were observed with Olympus Vanox light microscope.

TEM

The same samples were used as prepared for light microscopy except that sections, less than 100 nm thickness were stained using the PATAg reaction according to Thiéry (1967) for polysaccharide localization. Sections were observed with TEM (JEOL 100S) at 80 keV .

SEM

Caecal contents were washed with deionised water, then water was substituted with acetone after enzymic activity was stopped by sodium merthiolate. Samples were then critical point dried. After drying, samples were fixed on brass stubs with carbon glue then ion sputtered (gold deposit 25 nm). Bulk samples were observed with SEM (JEOL 840A) at 10 keV .

RESULTS

Establishment of bacterial strains in gnotobiotic rats

Bacteria were highly established in all groups: the amounts of bacteria found in the faeces of gnotobiotic rats were in the range of $5 \times 10^9 \text{ g}^{-1}$ in GNE rats,

$2 \times 10^{10} \text{ g}^{-1}$ in GNP rats, and 4.4×10^{10} *Eubacterium*, 5.9×10^9 *Peptostreptococcus*, 6.5×10^{10} *Enterococcus* and 5.5×10^9 *Veillonella* g^{-1} of faeces in GNT rats.

Digestibility of starch

In GF and GNP rats, food intake was similar: 17.8 ± 0.5 and $17.0 \pm 0.6 \text{ g day}^{-1}$ respectively; starch excretion (3.3 and 3.0 g day^{-1}) and digestive utilization of starch (67.9 and 69.7%), were not significantly different (Table 1). GNE rats fed $16.1 \pm 0.6 \text{ g}$ of diet per day but excreted significantly less starch than GF or GNP rats (0.7 g day^{-1}). The considerable improvement of digestive utilization of starch (from 68 to 92%) in rats associated with the amylolytic bacteria was more pronounced in the GNT group whose starch degradation was increased up to 97% (Table 1) without significant difference in food intake ($16.0 \pm 0.7 \text{ g day}^{-1}$).

Amylase activity

Amylase activity was similar in GF and GNP groups. It was significantly higher in GNE and GNT rats (36 and 54% , respectively) than in GF rats (Table 1).

Fermentation metabolites (Table 2)

In caecal content of GF rats, acetate concentration was very low ($2.5 \mu\text{mol g}^{-1}$) and from endogenous origin. No other metabolite was detected.

In the contents of GNP rats, SCFA concentration was $6.1 \mu\text{mol g}^{-1}$ ($5.8 \mu\text{mol}$ of acetate and $0.3 \mu\text{mol}$ of propionate); L-lactic and D-lactic acids (3.1 and $0.5 \mu\text{mol g}^{-1}$, respectively), and a low concentration of succinic acid ($0.4 \mu\text{mol g}^{-1}$) were found.

In GNE rats, amylolytic strain induced an enhancement of the SCFA concentration to $12.8 \mu\text{mol}$, especially due to propionate production whose concentration ($6.3 \mu\text{mol g}^{-1}$) reached the acetate concentration ($6.5 \mu\text{mol g}^{-1}$); respective concentrations of L-lactic and succinic acids were half time lower than in caecum of GNP rats.

Association of the four bacteria strains in GNT rats led to an increase of SCFA (to $29 \mu\text{mol g}^{-1}$) and succinic acid (to $2 \mu\text{mol g}^{-1}$) and a change of metabolites profile: propionic acid became predominant and D-lactic acid disappeared.

Ultrastructure of amylo maize starch granules

In the diet (Fig 1a), starch granules (purple stained by iodine) appeared more or less spherical with light

TABLE 1
Digestibility of amylo maize starch in germ-free rats (GF), in rats associated with *Peptostreptococcus* (GNP), with *Eubacterium* (GNE) and with four bacteria (GNT) (mean $\pm$ SD, $n = 6$)^a

Group	Starch in faeces (g day^{-1})	Digestive utilization (%)	Amylase activity (U g^{-1})
GF	$3.3 \pm 0.4 \text{ a}$	$67.9 \pm 1.7 \text{ a}$	$208 \pm 5 \text{ a}$
GNP	$3.0 \pm 0.3 \text{ a}$	$69.7 \pm 0.9 \text{ a}$	$207 \pm 3 \text{ a}$
GNE	$0.7 \pm 0.1 \text{ b}$	$92.2 \pm 1.5 \text{ b}$	$283 \pm 4 \text{ b}$
GNT	$0.31 \pm 0.03 \text{ c}$	$96.7 \pm 0.7 \text{ c}$	$321 \pm 4 \text{ c}$

^a The same following letters in the same column indicate no statistically significant difference ($P < 0.05$).

TABLE 2
Caecal concentration of acetate and other metabolites ($\mu\text{mol g}^{-1}$) in germ-free rats (GF) and rats associated with *Peptostreptococcus* (GNP), with *Eubacterium* (GNE) and with four bacteria (GNT) (mean $\pm$ SD, $n = 6$)^a

Group	Short-chain fatty acids				
	Acetate	Propionate	L-Lactic acid	D-Lactic acid	Succinic acid
GF	$2.5 \pm 0.2 \text{ a}$	0	0	0	0
GNP	$5.8 \pm 0.2 \text{ b}$	$0.30 \pm 0.03 \text{ a}$	$3.1 \pm 0.2 \text{ a}$	$0.5 \pm 0.3 \text{ a}$	$0.40 \pm 0.06 \text{ a}$
GNE	$6.5 \pm 0.2 \text{ b}$	$6.3 \pm 0.8 \text{ b}$	$1.8 \pm 0.2 \text{ b}$	$0.7 \pm 0.4 \text{ a}$	$0.17 \pm 0.05 \text{ b}$
GNT	$13.0 \pm 0.3 \text{ c}$	$16.0 \pm 0.9 \text{ c}$	$2.9 \pm 0.2 \text{ a}$	0	$1.95 \pm 0.09 \text{ c}$

^a The same following letters in the same column indicate no statistically significant difference ($P < 0.05$).

Fig 1. Light microscope photographs of (a) diet; (b) caecal contents of GF rats; (c) caecal contents of GNP rats; (d) caecal contents of GNE rats. Casein (c) appears light grey in the diet, discolours in GF and disappears in GNP and GNE samples. Cellulose fibres (f) seem poorly digested. Scale, 50 μ m.

central part; the biggest grey particles were casein (green stained by fast green); smallest and elongated particles were cellulose fibres. In caecal contents (Fig 1b–d) the starch granules lost their iodine-binding capability. In this case staining was due to the adsorption of fast green on starch granules. Moreover, differences were observed between groups: starch breakdown was more pronounced in GNE (Fig 1d) than in GF (Fig 1b) and GNP (Fig 1c) samples, GNE showing some peripheral parts of the starch granules and a large amount of starch ghosts. In addition, casein which appeared green

in the diet remained partly in the GF and disappeared in the other groups. Cellulose fibres seemed poorly digested in those intestinal contents.

Ultrastructure study (Figs 2 and 3): observations of the caecal contents of GF (Fig 2a) or GNP (Fig 2b) rats by transmission electron microscopy showed that amyloamylase starch granules were weakly hydrolyzed, as shown by endocorrosion in the central part of the granules. In the contents of GNE (Fig 2d) and GNT (Fig 2e) rats, bacteria were found all around the starch granules and some specific zones inside the peripheral part of the

Fig 2. Transmission electron micrographs of caecal contents after PATAg staining. (a) GF rats; (b) and (c) GNP rats; (d) and (f) GNE rats; (e) GNT rats. Scale, 1 μ m.

Fig 3. (a) and (b) Scanning electron micrographs of caecal contents of GNE rats; (c)–(f) transmission electron micrographs of caecal contents after PATAg staining; (c), (d) and (f) GNT rats; (e) GNE rats. Scale, 1 μ m.

granules (the most resistant part) showed pits due to endocorrosion. This outer part was almost entirely hydrolysed in GNT rats. At higher magnification, radial microporosity which can be seen in GNP (Fig 2c) was more pronounced in rats associated with amylolytic bacteria (Fig 2f).

Figures 3a and 3b are SEM views of GNE caecal contents. Figure 3a shows apparently undamaged starch granules with many bacteria laying in the intestinal mucus. On the starch surface are seen small protuberances with one pore on the top. In the same sample only few granules are broken, as in Fig 3b,

showing that the inside can be more or less hydrolysed while the peripheral part may appear unchanged. Much more information can be obtained from TEM observations, owing to better resolution and specific chemical groups (α -glycols) staining in ultrathin sections. Figures 3c–3f showed different steps of bacterial breakdown. Observations show: (i) a progressive hydrolysis of the inside of the granules by bacteria (Fig 3c) then a peripheral digestion with bacteria penetrating the granules by enlarged pores (Fig 3d); and (ii) during digestion processing, some starch granules partly hydrolysed may be broken by the gastrointestinal mobility. In that case

(Figs 3e and 3f) hydrolysis occurs simultaneously on all parts.

DISCUSSION

In GF rats, 30% of the amylomaize starch escaped endogenous digestion. This confirms previous results, on normal maize starch (30% amylose) which was totally degraded compared with amylomaize starch (70%) in germ-free rats (Andrieux and Sacquet 1986), or in rats treated with antibiotics (Grandfeldt *et al* 1993). In humans, analysis of the ileal content shows that retrograded amylomaize starch is very resistant to endogenous amylolysis, and exhibits a B-type pattern by X-ray diffraction, as does uncooked amylomaize starch (Faisant *et al* 1993).

In uncooked starches, large variations in the susceptibility of the starch granules to enzymic digestion are dependent on the morphology of the granules and their crystalline organisation (Gallant *et al* 1992). Most of the starch granules are first hydrolysed superficially by endocorrosion, a type not seen with amylomaize starch except after treatment with α -amylase from *Aspergillus* sp K-27 of which granules presented a rough scratched surface (Planchot 1993). As already described, a very resistant outer shell protects the amylomaize starch granule against chemical or enzymic degradation (Gallant *et al* 1973, 1992; Gallant and Bouchet 1986). Thickness of this outer shell varies from 1 to 2 μm and the shell is constituted by very large and dense blocklets made of crystalline material (Gallant 1974) closely packed together. So that porosity of such starch granules is low. When porcine pancreatic α -amylase acts, few local pores randomly distributed all around the granules may occur, starting by a small local swelling at granule surface and giving rise to protuberances with one pore on the top. Enzyme penetrates through these pores and hydrolyses progressively the starch content, the amylomaize starch granule remaining undigested externally. On sample sections we observed that the content of the amylomaize starch granule can be totally emptied.

New results obtained *in vitro* with α -amylase of *Aspergillus* sp K-27 on amylomaize starch showed the same phenomenon but with more external degradation and larger pores (Planchot 1993).

The *in-vivo* degradation we observed appears to be quite similar to the *in-vitro* attack by pig pancreatic α -amylase observed by Gallant *et al* 1973. We previously observed (Andrieux *et al* 1992) that endogenous amylase was soluble in the caecal content but mainly fixed to the starch granules. This binding could explain that starch granules lost their iodine-binding capability and appeared green, as undigested proteins, in light microscopy. In GNP rats associated with the non-amylolytic *Peptostreptococcus*, no change in the

digestive utilization was observed. The same results were obtained when germ-free rats were mono-associated with the two non-amylolytic strains: *Enterococcus* or *Veillonella* (Pacheco Delahaye 1990). By contrast, in GNE rats, *Eubacterium* led to the increase of amylase activity (36%) and subsequent decrease of starch concentration (38%) in the caecum. In these rats we identified four enzymes contributing to the *in-vivo* amylomaize starch hydrolysis: the three endogenous enzymes (salivary and pancreatic α -amylases, and maltase) and one bacterial α -amylase whose characteristics (pI, molecular weight and optimum pH activity) differ from those of rat endogenous amylases (Pacheco Delahaye *et al* 1991). This bacterial enzyme seems able to improve the peripheral shell degradation and the endocorrosion of the starch granules whose breakdown were more pronounced in the caecum of GNE compared with GF rats. Hydrolysis *in vivo* by only one strain (B86) gives the same results as described previously *in vitro*, but association of two or four strains increases the action of bacteria. Acting by synergy the four bacterial strains are also able to hydrolyse totally the external part of the amylomaize starch granule. In GNT rats, association of *Eubacterium* with the other bacteria, which alone are not able to hydrolyse amylomaize-resistant starch, induced an increase of amylase activity and of starch digestive utilisation. In GNT rats, compared with GNE rats, the concentration of bacterial metabolites was double and fermentation profile was modified: D-lactic acid disappeared and succinic acid increased. This increase of bacterial metabolites production suggests that the non-amylolytic strain's contribution to starch degradation could be related to the capability of the bacteria to ferment the end-products of the starch hydrolysis by the *Eubacterium*. Thus, *Veillonella* is able to hydrolyse and ferment maltose and maltodextrins, and to utilize lactic acid; *Enterococcus* ferments maltose producing a high level of lactic acid and *Peptostreptococcus* fermented glucose which is the highest reducing sugar component in the caecum of germ-free rats (Pacheco Delahaye 1990).

Many other amylolytic bacteria should be able to participate in amylomaize degradation. We chose the most efficient amylolytic bacteria among the 18 colonies able to degrade resistant starch, and other amylolytic bacteria have been previously isolated from dominant genera of the conventional rat (Pacheco Delahaye 1990). In the caecum of conventional rats, no trace of starch granules was found (Andrieux *et al* 1992) suggesting a very fast hydrolysis of the resistant starch granules. Moreover, the fermentability of resistant starches, from raw or cooked starches, appears very high in rats as shown by metabolites analysis such as SCFA or H_2 excretion (Mallett *et al* 1988; Andrieux *et al* 1992; Björck and Siljeström 1992; De Deckere *et al* 1993). From human stools, Macfarlane and Englyst (1986) isolated

120 starch-hydrolysing colonies belonging to the genera: *Bifidobacterium*, *Bacteroides*, *Fusobacterium* and *Butyrivibrio*, and Breath test studies have shown that fermentation of resistant starches was also high in human (Fluorié *et al* 1988; Olesen *et al* 1992).

CONCLUSION

These results confirm endogenous and bacterial contribution to the in-vivo amylomaize starch degradation and show, in gnotobiotic models, that an association of bacterial strains improves starch degradation. They suggest that resistant starch breakdown in the hindgut is not only due to amylolytic bacteria but also to the contribution of other glycolytic bacteria able to utilize the end-products of starch hydrolysis.

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