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Digestion of Barley, Maize, and Wheat by Selected Species of Ruminal Bacteria†

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Differences in the digestion of barley, maize, and wheat by three major ruminal starch-digesting bacterial species, *Streptococcus bovis* 26, *Ruminobacter amylophilus* 50, and *Butyrivibrio fibrisolvens* A38, were characterized. The rate of starch digestion in all cereal species was greater for *S. bovis* 26 than for *R. amylophilus* 50 or *B. fibrisolvens* A38. Starch digestion by *S. bovis* 26 was greater in wheat than in barley or maize, whereas starch digestion by *R. amylophilus* 50 was greater in barley than in maize or wheat. *B. fibrisolvens* A38 digested the starch in barley and maize to a similar extent but was virtually unable to digest the starch in wheat. The higher ammonia concentration in cultures of *B. fibrisolvens* A38 when grown on wheat than when grown on barley or maize suggests that *B. fibrisolvens* A38 utilized wheat protein rather than starch. Scanning electron microscopy revealed that *B. fibrisolvens* A38 initially colonized cell wall material, while *S. bovis* 26 randomly colonized the endosperm and *R. amylophilus* 50 preferentially colonized starch granules. There was subsequent colonization but only superficial digestion of wheat starch granules by *B. fibrisolvens* A38. Variation in the association between starch and protein within the endosperm of cereal grains contributes to the differential effectiveness with which amylolytic species can utilize cereal starch.

Starch is the major source of energy in the diets of high-producing ruminants. Consequently, researchers have attempted to determine which bacteria are responsible for starch digestion in the rumen. Pure culture techniques have been used to isolate several species of starch-fermenting bacteria (25). Much attention has been focused on the amylolytic activity of *Streptococcus bovis* (27, 28), which has been implicated in the development of lactic acidosis in ruminants (22). Few researchers have attempted to determine the relative contribution of different amylolytic bacteria to starch digestion within the rumen. Several species of amylolytic bacteria have recently been screened (4), and *S. bovis* JB1 and *Ruminobacter amylophilus* H18 were found to exhibit the highest levels of amylase activity, followed by *Butyrivibrio fibrisolvens* A38 and 49 and *Bacteroides ruminicola* B14 and 23. Researchers (13) have shown that ruminal bacteria which attach to starch have a higher specific amylase activity than unattached bacteria. All these studies have utilized isolated starch or starch granules to characterize the amylolytic activity of ruminal bacteria.

Starch is fed to ruminants in the form of cereal grains. Cereal grains are complex in structure in that the interior contains endosperm cells, which consist of a cell wall surrounding starch granules embedded within a protein matrix (16). The extent to which pure cultures of ruminal bacteria digest isolated starch may not accurately reflect their ability to digest starch in structurally complex cereal grains. In addition, we have demonstrated that cereal grains differ in their susceptibility to microbial attack and that the bacteria responsible for the digestion of barley starch are morphologically different from those involved in the digestion of maize starch (11c, 11d). Therefore, the type of cereal grain may influence the relative ability of an amylolytic bacterial species to ferment starch within the rumen. The objective of this study was to examine the ability of three of the major species of amylolytic ruminal bacteria to digest starch in the endosperm of barley, maize, and wheat kernels.

MATERIALS AND METHODS

The organisms used were the amylolytic species *R. amylophilus* 70 (formerly *Bacteroides amylophilus* [23]), *B. fibrisolvens* A38, and *S. bovis* 26. Amylolytic bacteria were grown anaerobically in batch culture in the liquid medium of Scott and Dehority (21) with 0.2% starch as the sole source of carbohydrate. The cultures were transferred every 1 to 2 days.

Barley, maize, and wheat were ground and sieved to obtain a particle size between 0.85 and 2 mm. Grains were sterilized by exposure to radiation for 50 h resulting in a total dose of 1,500 krads (11b). Grains (0.5 g) were added aseptically to 30-ml quadruplicate vials along with anaerobic liquid medium (20 ml) containing no starch. Cultures were transferred to liquid medium with 0.2% starch (300 ml), and growth was monitored spectrophotometrically (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) by determining the optical densities of cultures at 600 nm. Bacterial numbers were estimated by the anaerobic plate culture method as described by Ogimoto and Imai (15). Cultures were used for inoculation when the optical density reached a level corresponding to 1×10^8 cells ml^{-1}. Vials were inoculated with 2 ml of *R. amylophilus*, *B. fibrisolvens*, *S. bovis*, or liquid medium (control) and were incubated anaerobically (under an initial CO_2 atmosphere) in a shaking incubator at 39°C for 0, 4, 8, 12, 24, 48, and 72 h. Gas production in quadruplicate vials was determined by using a water displacement apparatus (7). The pH was measured, and microbial digestion was stopped by adding 2 ml of 2 N HCl to each vial. Samples were stored at −40°C until analyzed in triplicate for starch and ammonia.

The anaerobic technique of Hungate (10), as modified by Bryant and Burkey (2), was used throughout the experiment.

Starch was analyzed by a modified method of MaCrae and

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Armstrong (11). Undigested grain particles remaining after incubation were homogenized with a Polytron tissue homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada) for two 20-s periods. Samples were neutralized by the addition of 2 ml of 2 N NaOH. Vials were capped, and the starch was gelatinized by autoclaving at 130°C for 90 min. Samples were cooled, and while stirring, a 0.5-ml portion was added to 0.5 ml of 0.2 M acetate buffer (pH 4.5) in test tubes (13 by 100 mm). Amyloglucosidase (Boehringer Mannheim, Dorval, Quebec, Canada) was suspended in 0.2 M acetate buffer (pH 4.5) at an activity of 20 U ml^{-1}, and 1 ml of this solution was added to the sample-acetate buffer mixture. Tubes were capped and incubated for 24 h at 60°C. The glucose arising from hydrolysis was immediately determined by a glucose oxidase assay (Sigma Chemical Co., St. Louis, Mo.).

Ammonia concentration in the vials was determined by the phenol-hypochlorite method as described by Weatherburn (30). Digested grain samples from the fourth replicate vial were fixed for 3 h in 5% glutaraldehyde in 0.1 M cacodylate (pH 7.2). Samples were washed (five times) in sodium cacodylate buffer, dehydrated in a graduated ethanol series, and critical-point dried (3). Specimens were mounted on aluminum stubs with silver paste and sputter coated with gold. Specimens were viewed with a Hitachi S-570 scanning electron microscope (SEM) at an accelerating voltage of 7 kV and photographed with Kodak T-Max film.

Data were analyzed by using the general linear model procedure of the SAS Institute (24). Differences among bacteria and among cereal grains were compared by orthogonal contrasts. The least-square means procedure was used to determine differences at each time point.

RESULTS

The digestion of barley, maize, and wheat starch by ruminal bacteria is illustrated in Fig. 1. *S. bovis* 26 digested the starch in all three cereal grains more rapidly than *R. amylophilus* 70 or *B. fibrisolvens* A38. *S. bovis* 26 digested wheat more ($P < 0.05$) than maize or barley after 48 h. The digestion of barley by *R. amylophilus* 70 was greater ($P < 0.001$) than that of maize or wheat after 12 h. Orthogonal contrasts indicated that there was no difference between the digestion of maize and wheat by *R. amylophilus* 70 ($P > 0.05$). When compared with *S. bovis* 26 and *R. amylophilus* 70, *B. fibrisolvens* A38 digested the greatest percentage of starch in barley and maize in 72 h of incubation. In contrast, by 72 h, *B. fibrisolvens* A38 had digested the lowest percentage of wheat when compared with the other two amylolytic species. The digestion of wheat by *B. fibrisolvens* A38 was lower ($P < 0.01$) than that of barley and maize after 12 and 24 h, respectively.

In most instances, ammonia concentration showed a gradual decline or no change over the incubation period (Fig. 2). In general, the ammonia concentration in media with *B. fibrisolvens* A38 was higher than in *S. bovis* 26 and *R. amylophilus* 70 with all cereal grains. Cultures of *R. amylophilus* 70 tended to have lower ammonia concentrations than either *S. bovis* 26 or *B. fibrisolvens* A38. There was no significant ($P > 0.05$) difference in the ammonia concentration of cultures of *S. bovis* 26 grown on barley, maize, or wheat. The ammonia concentration in cultures of *B. fibrisolvens* A38 grown on wheat increased throughout the experiment. After 12 h of incubation, the ammonia concentration in cultures of *B. fibrisolvens* A38 grown on wheat was greater ($P < 0.01$) than in those grown on barley, while after

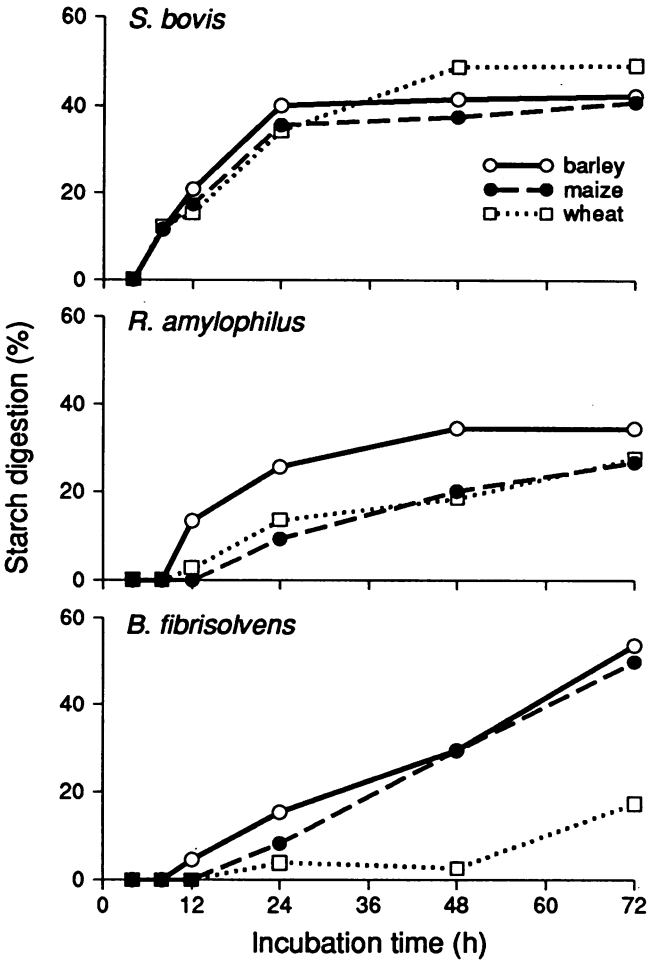

FIG. 1. Percentage of starch digested in barley, maize, and wheat by *S. bovis* (standard error [SE] is ±1.44), *R. amylophilus* 70 (SE is ±1.49), and *B. fibrisolvens* A38 (SE is ±1.96).

24 h, it also exceeded ($P < 0.01$) that in cultures grown on maize.

Decline in culture pH as a result of acid production by amylolytic bacteria (Fig. 3) was closely linked to starch digestion. There was a rapid decline in the pH of cultures of *S. bovis* 26 in comparison with *R. amylophilus* 70 and *B. fibrisolvens* A38, regardless of the species of cereal grain. As a result, for all three cereal grains, pH was lower at all times in cultures of *S. bovis* 26 than in cultures of *R. amylophilus* 70 or *B. fibrisolvens* A38 (Fig. 3). The pH of *S. bovis* 26 cultures grown on barley was lower ($P < 0.01$) than the pH of those grown on maize after 4 h of incubation. Although the pH of barley cultures was lower ($P < 0.01$) than that of wheat cultures between 8 and 24 h, these pH measurements did not differ after 48 h of incubation with *S. bovis* 26. There was no difference ($P > 0.05$) in the pH of cultures of *R. amylophilus* 70 incubated with maize or wheat. The pH of *R. amylophilus* 70 grown on barley was lower ($P < 0.01$) than those of cultures grown on wheat and maize after 8 h and 24 h of incubation, respectively. The pH of wheat cultures showed only a slight decline over the period of incubation with *B. fibrisolvens* A38. The pH of *B. fibrisolvens* A38 cultures grown on barley was lower ($P < 0.01$) than that of those grown on wheat and maize after 12 h, and after 24 h,

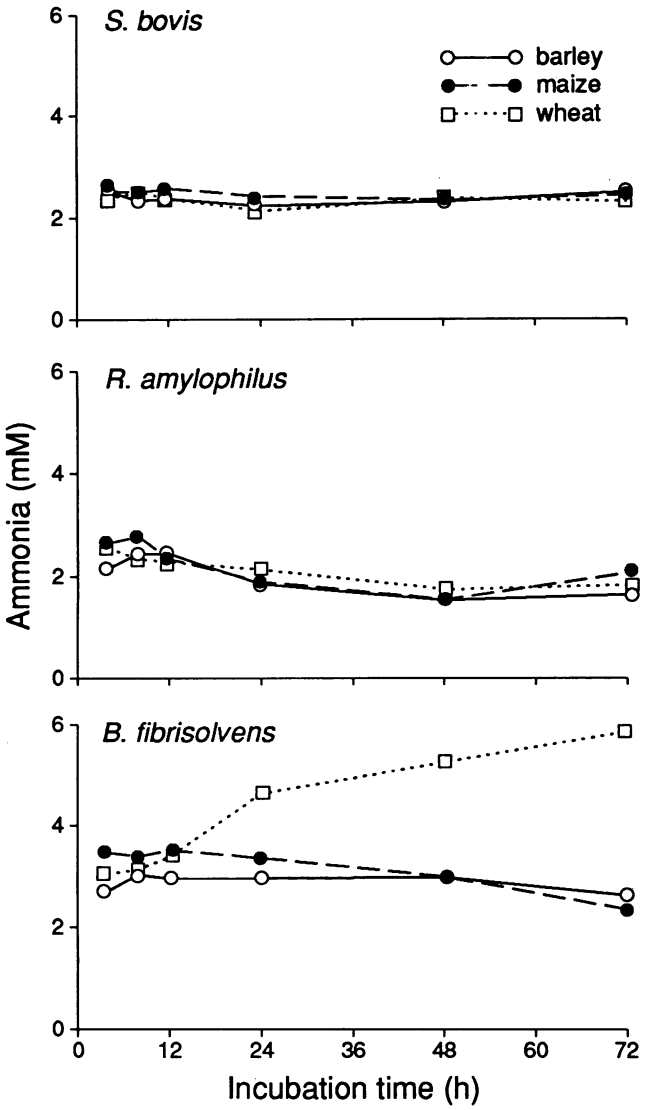

FIG. 2. Ammonia concentration in cultures of *S. bovis* 26 (SE is ±0.11), *R. amylophilus* 70 (SE is ± 0.13), and *B. fibrisolvens* A38 (SE is ±0.16) grown on barley, maize, and wheat.

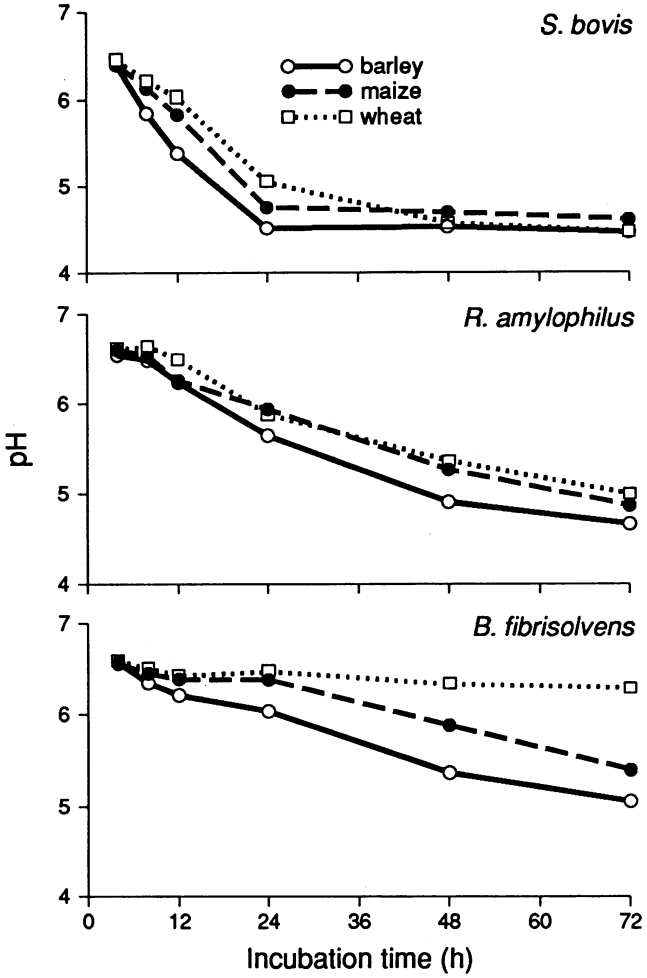

FIG. 3. pH of cultures of *S. bovis* 26 (SE is ±0.026), *R. amylophilus* 70 (SE is ±0.051), and *B. fibrisolvens* A38 (SE is ±0.031) grown on barley, maize, and wheat.

the pH in cultures containing maize was lower ($P < 0.05$) than that in those containing wheat.

In general, *B. fibrisolvens* A38 produced the most gas, followed by *S. bovis* 26 and *R. amylophilus* 70 (Fig. 4). The exception to this pattern occurred in wheat, with which *B. fibrisolvens* A38 and *S. bovis* 26 exhibited similar levels of gas production. Gas production by *S. bovis* 26 when incubated with barley was greater ($P < 0.01$) than that with maize and wheat between 8 and 24 h, whereas amount of gas produced did not differ between maize and wheat. *R. amylophilus* 70 produced similar levels of gas regardless of the type of cereal grain. Gas production by *R. amylophilus* 70 incubated with barley was greater ($P < 0.05$) than that with wheat or maize at 12 and 24 h, respectively. After 4 h, gas production by *B. fibrisolvens* A38 cultures grown on barley was greater ($P < 0.01$) than that of those grown on maize or wheat, while gas production with maize was greater ($P < 0.01$) than that with wheat after 24 h.

Examination by SEM showed that initial colonization of starch granules by *S. bovis* 26 occurred at 4 h of incubation (Fig. 5). With *R. amylophilus* 70, similar patterns of colonization of starch granules were not observed until after 12 h of incubation (Fig. 6). *S. bovis* 26 appeared to randomly colonize the endosperm, while *R. amylophilus* 70 appeared to preferentially colonize starch granules as opposed to other regions of the cereal endosperm. In contrast, initial colonization (4 h) of the endosperm by *B. fibrisolvens* A38 appeared to occur on cell wall material (Fig. 7). Although some colonization of starch granules by *B. fibrisolvens* A38 was seen, the preferential colonization of cell wall material was still evident in maize even after 24 h of incubation (Fig. 8). Colonization of wheat starch granules by *B. fibrisolvens* A38 was noted in the later stages of incubation (72 h), but these structures never showed evidence of extensive amylolytic digestion (Fig. 9). After 24 h of incubation with *S. bovis* 26, the endosperm of cereal grains was not as heavily colonized as in earlier preparations and in maize the starch granules in most horny endosperm cells were removed, leaving behind an intact protein matrix (Fig. 10). Further observations indicated that the protein matrix in the endosperm cells of maize was equally resistant to digestion by *R. amylophilus* 70 and *B. fibrisolvens* A38.

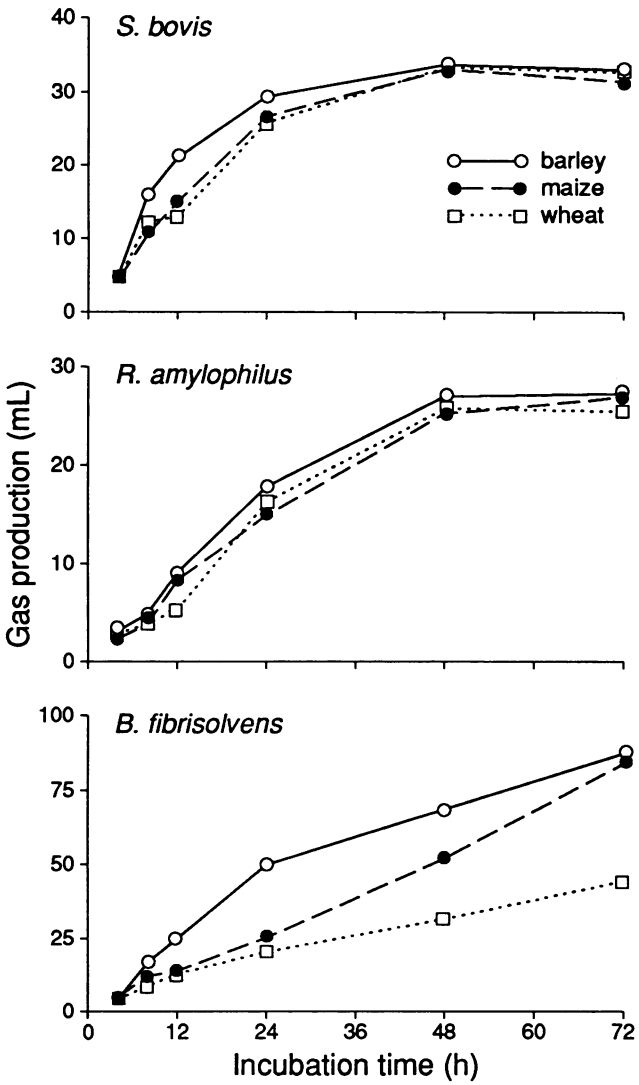

FIG. 4. Gas production by *S. bovis* 26 (SE is ±0.66), *R. amylophilus* 70 (SE is ±0.59), and *B. fibrisolvens* A38 (SE is ±1.36) grown on barley, maize, and wheat.

Digestion patterns, indicated by pits on starch granules, differed between cereal grains. In wheat and barley, patterns of amylolytic digestion tended to spread outward on the surface of the granule from a central point (Fig. 11). In contrast, the pattern of amylolytic attack in maize was more focused and did not spread over the surface of starch granules (Fig. 12). Bacteria appeared to gain access to and digest the interior of the starch granule, resulting in the formation of cavities (Fig. 12).

DISCUSSION

Pure cultures of amylolytic bacteria exhibit differential abilities to digest cereal starches, which depend at least in part on the species of cereal grain. The rate of starch digestion by *S. bovis* 26, which is reported to be a rapidly growing species (19), was substantially greater than the starch digestion rates of either *R. amylophilus* 70 or *B. fibrisolvens* regardless of cereal species; however, *R. amylophilus* 70 fermented barley starch more rapidly and to a greater extent than either maize or wheat, and *B. fibrisolvens* A38, while digesting barley and maize starch similarly, was virtually unable to utilize wheat starch.

Changes in culture pH were closely linked to the extent of starch digestion. The rapid decline in the pH of *S. bovis* 26 cultures is likely a result of greater volatile fatty acid production and the accumulation of lactate (18). As the pH declined to below 4.8, the utilization of cereal starch by *S. bovis* 26 was inhibited. This point of inhibition is in close agreement with the results of other researchers (26), who found that growth of *S. bovis* ceased at a pH of 4.6. Additionally, researchers (26) found that the lower limit for growth of *B. fibrisolvens* occurred at a pH of 5.4. In the present study, a pH below 5.4 was only achieved when *B. fibrisolvens* A38 was incubated with barley. The absence of a plateau in starch digestion or gas production indicates that barley starch fermentation continued at a pH below 5.4. Utilization of barley starch by *R. amylophilus* 70 appeared to be inhibited when the pH declined below 5.0 as indicated by the plateau in starch digestion. In contrast, when this strain was incubated with maize and wheat, medium pH did not drop below 5.0 and a plateau in starch digestion was not obtained. Further research is required to determine the effects of pH on bacterial attachment and starch digestion.

Ammonia concentrations showed little change when cereal grains were supplied as substrates for *S. bovis* 26 or *R. amylophilus* 70. Strains of *S. bovis* are capable of proteolysis (17) and amino acid deamination (20). However, in agreement with our work, other researchers have found that ammonia concentrations remained constant when *S. bovis* was incubated with glucose and casein (17). The lack of an increase in ammonia concentration in *R. amylophilus* 70 cultures is not surprising, since strains of this bacterium are unable to utilize peptides or amino acids (9). Ammonia concentration in the cultures increased when *B. fibrisolvens* A38 was incubated with wheat. Researchers (5, 29) have shown that *Butyrivibrio* spp. exhibit a high proteolytic activity and are capable of amino acid deamination (1). In the present study, the increase in ammonia concentration suggests that *B. fibrisolvens* A38 is capable of proteolysis and amino acid deamination.

In this study, the association of bacteria with the endosperm was seen by SEM to differ among amylolytic bacteria. *S. bovis* 26 appeared to associate randomly with the endosperm of cereal grains and was observed on both starch granules and the protein matrix. Several strains of *S. bovis* are unable to attach to isolated starch granules (14). However, it has been shown that when it is grown on starch, most of the amylolytic activity exhibited by *S. bovis* JB1 is cell associated (4). Presumably, *S. bovis* JB1 must be in close proximity to starch granules for cell-associated amylases to digest starch effectively. Unlike *S. bovis* 26, the association of *R. amylophilus* 70 with the endosperm was not random, and this strain was observed to preferentially colonize cereal starch granules after 12 h. Similarly, other researchers have found that several strains of *R. amylophilus* rapidly colonize isolated maize starch granules (14). The amylolytic activity of *B. fibrisolvens* A38 is largely cell associated (4). However, unlike *R. amylophilus*, most strains of *B. fibrisolvens* appear to be incapable of attaching to starch granules (14). In the present study, *B. fibrisolvens* A38 appeared to preferentially colonize cell wall regions as opposed to other areas of the endosperm. After 24 h of incubation, digestion of cereal starch granules was observed but the preferential colonization of cell wall material by *B. fibrisolvens* A38 was still apparent.

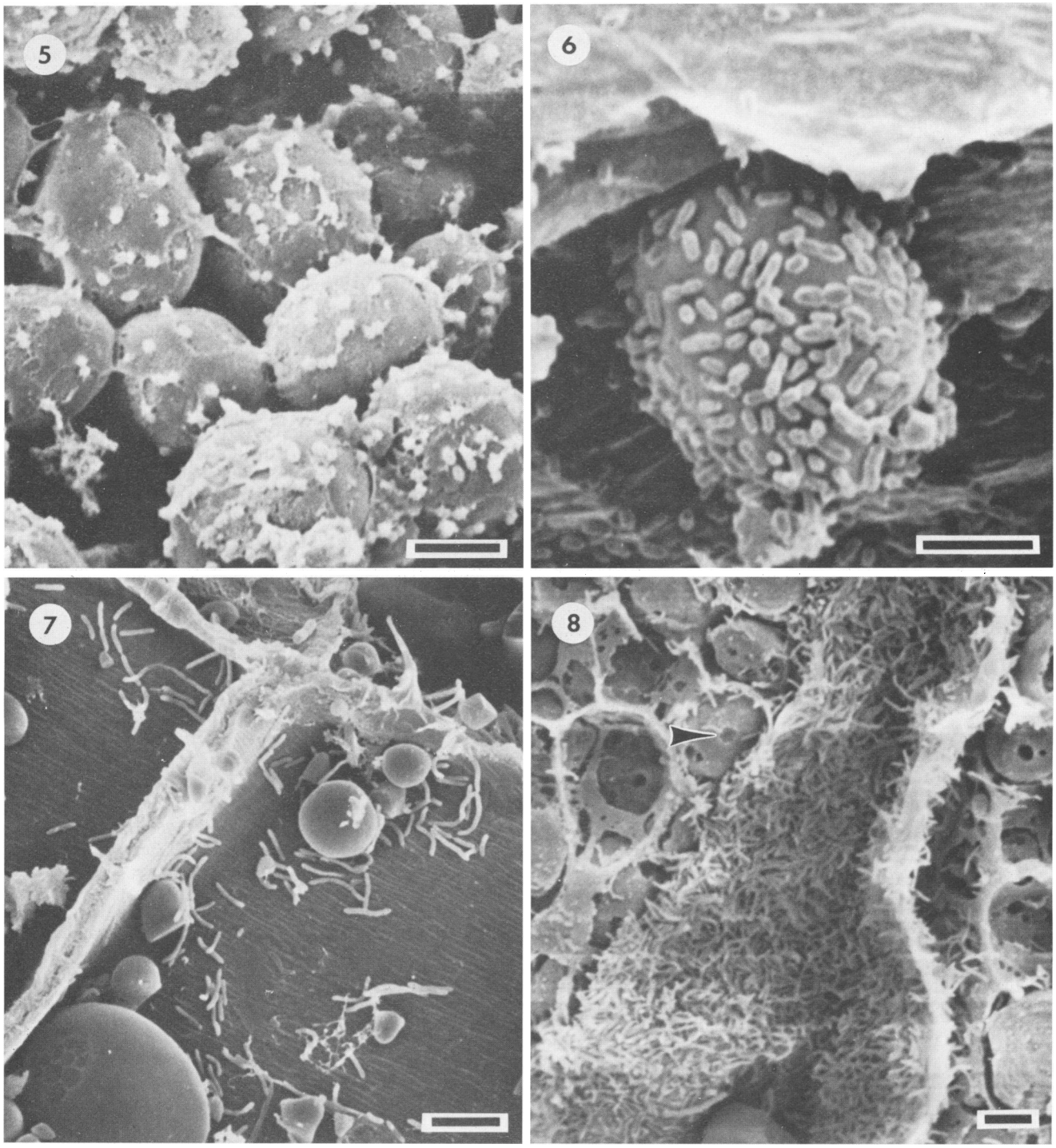

FIG. 5. SEM of maize starch granules after 4 h of incubation. Note the initial colonization of starch granules by *S. bovis* 26. Bar = 5 μm.

FIG. 6. SEM of *R. amylophilus* 70 attached to a wheat starch granule after 12 h of incubation. Note that bacteria have preferentially colonized the granule rather than other regions of endosperm. Bar = 5 μm.

FIG. 7. SEM of *B. fibrisolvens* A38 attached to cell wall region in wheat. Note that starch granules do not appear to be the initial site of colonization. Bar = 5 μm.

FIG. 8. SEM of *B. fibrisolvens* A38 attached to endosperm cell in maize. Although digestion of starch granules is apparent (arrowhead), *B. fibrisolvens* A38 appears to preferentially colonize the cell wall region. Bar = 5 μm.

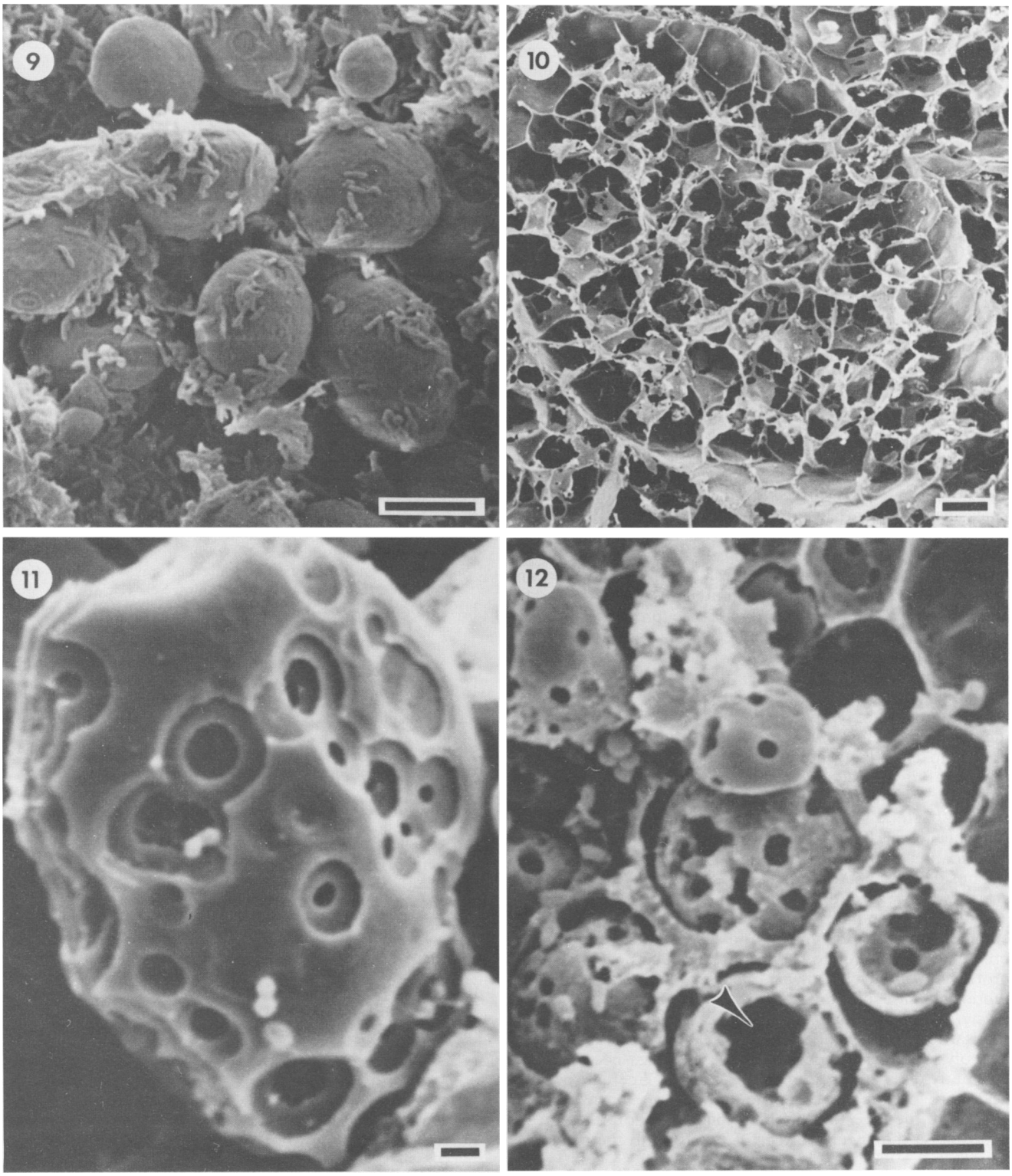

FIG. 9. SEM of *B. fibrisolvens* A38 attached to the starch granules in wheat. Note that despite bacteria attachment, starch granules do not show evidence of extensive amylolytic digestion. Bar = 5 μm.

FIG. 10. SEM of maize endosperm cell after 12 h of incubation with *S. bovis* 26. Note that starch granules have been removed from the cell, while the protein matrix is still intact. No bacterial colonization is evident. Bar = 10 μm.

FIG. 11. SEM of wheat starch granule after 72 h of incubation with *S. bovis* 26. Patterns of amylolytic digestion appear to spread outward on the surface of the granule from a central point of bacterial attachment. Bar = 1 μm.

FIG. 12. SEM of maize starch granules after 24 h of incubation with *R. amylophilus* 70. Note that sites of amylolytic attack are more focused than those of Fig. 10 and that the interiors of the granules have been digested (arrowhead). Bar = 5 μm.

Structural properties of the starch granule, along with the hydrolytic characteristics of the amylases, contribute to the digestive patterns observed on starch granules. *S. bovis* and *R. amylophilus* possess both cell-associated and extracellular amylases (4, 14). The cratered patterns of amylolytic digestion of wheat and barley starch granules suggest that cell-associated amylases result in a concentrated digestion of the starch granule at the site of bacterial attachment. Higher amylase activity at these sites is consistent with previous work (27) which demonstrated that cell-associated amylolytic enzymes have a greater activity than extracellular amylases. Alternatively, these patterns of digestion may arise from a gradual dilution of the extracellular amylolytic activity as the distance from the bacterial cell increases.

Patterns of amylolytic digestion on maize starch granules differed from those on barley and wheat starch granules. Amylolytic attack on maize starch granules appeared to be more focused, and the cratered digestion pattern commonly seen on wheat and barley starch granules was not observed. In agreement with previous work (8), digestive patterns on maize starch granules suggest that the surface layer of these granules is more resistant than the interior to amylolytic digestion. In the present study, amylolytic bacteria appeared to gain access to the interior of maize starch granules by digesting through the surface layer via the most direct route. Once inside, bacteria were able to effectively ferment the interior components. The greater resistance to amylolytic attack of the surface layer of maize starch granules compared with wheat and barley starch granules may explain why maize starch is more resistant than wheat or barley starch to fermentation even when the properties of the protein matrix have been destroyed by fine grinding.

Earlier work from our laboratory has shown that the horny endosperm region of maize is extremely resistant to microbial colonization and digestion (11d). In the present study, pure cultures of amylolytic bacteria were capable of digesting starch granules within the horny endosperm but were unable to effectively attack the protein matrix. A similar pattern of digestion was previously observed when formaldehyde-treated barley was incubated with mixed cultures of ruminal bacteria (11a). In contrast to maize, all three bacterial species in this study were capable of digesting the protein matrix in barley and wheat.

Considerable anatomical differences exist among cereal grains with regard to starch granule and protein matrix structure. The complete digestion of cereal grains requires a complex array of enzymes. The fact that several of the predominant amylolytic bacteria (4, 12) also possess a high proteolytic activity (1, 6, 17) is not merely coincidence but represents an evolutionary adaptation which is vital for the efficient fermentation of cereal grains. However, despite this adaptation, it is highly unlikely that any one particular amylolytic bacterium possesses the enzymes necessary to efficiently digest the starch and protein in all cereal species. Therefore, the relative contribution of any one bacterial species to the digestion of starch and protein in the rumen depends on the species of cereal grain fed to the ruminant animal.

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