Chapter 5

Synbiotic Matrices Derived from Plant Oligosaccharides and Polysaccharides

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A porous synbiotic matrix was prepared by lyophilization of alginate and pectin or fructan oligosaccharides and polysaccharides cross-linked with calcium. These synbiotic matrices were excellent structures to support the growth of Lactobacillus acidophilus and Lactobacillus reuteri under anaerobic conditions. When the matrix was inoculated with lactobacilli and stored at 4°C under aerobic conditions for a month, bacterial viability was preserved even though the synbiotic dried to a hard pellet.

Functional foods that benefit health by including live friendly bacteria as ingredients have a long history of use in Asia and Europe, and are rapidly emerging in the US. Symbiotics are a combination of probiotic, or health-promoting bacteria, and a prebiotic. Probiotic bacteria, such as Bifidobacteria and Lactobacillus, are facultative anaerobes found in the gut that promote host health. Prebiotics are components of non-digestible fiber that selectively stimulate the growth and/or activity of one or a limited number of these probiotic bacteria (1). Native probiotic bacteria can become depleted due to antibiotic therapy or diets low in soluble fiber and rich in refined carbohydrates (2). Dietary manipulation of the colonic microflora using these agents can

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restore the probiotic microflora, prevent pathogen colonization through competitive exclusion, stimulate the immune system and may play a role in the prevention of gastrointestinal diseases. Unfortunately, long term colonization of the gut by orally administered probiotic bacteria has been difficult to demonstrate. Additionally, viability of probiotic bacteria in commercial products varies. Therefore, prebiotics that selectively stimulate the growth of native probiotic bacteria is an attractive strategy to improve gut microflora composition and consequently host health. Another alternative is to supply synbiotics such that the probiotic growth is enhanced as much as possible in the colon. Supplying lactobacilli in the presence of fructo-oligosaccharides (FOS) produced higher daily weight gain and better feed conversion ratio for swine compared to feeds supplemented with the probiotic alone (3).

Pectic oligosaccharides (POS) are known to have prebiotic properties (4, 5). Since guluronic acid is an epimer of galacturonic acid, and both alginate and pectin can be crosslinked with calcium, we examined alginic acid-calcium and POS as a synbiotic matrix. Modified citrus pectin (MCP) is a low molecular weight, low-methoxy pectin rich in oligogalacturonic acids (6). However, it is not known if MCP has prebiotic properties. FOS and inulin were included in alginic acid-calcium matrices as examples of currently used commercial prebiotics.

**Materials and Methods**

A solution of high viscosity alginic acid (Type IV, Sigma; 10 mg/ml) and another oligo/poly-saccharide (10 mg/ml) was prepared in deionized water. Other saccharides included orange peel pectic oligosaccharides (5), modified citrus pectin (Pectasol, EcoNugenics, Santa Rosa, CA), fructo-oligosaccharides (Raftilose P95, Orafti, Malvern, PA) and inulin (Raftilose Synergy1, Orafti, Malvern, PA). The solution was pipetted into a 96 well plate (120 µl/well) and then lyophilized. The dry matrix was immersed in a 0.5% CaCl2 solution for 20 minutes. The matrix was then washed with deionized water (3x, stirring). The calcium cross-linked matrix was then returned to the 96 well plate and lyophilized. Finally, the matrix was washed with ethanol.

Cultures of *Lactobacillus acidophilus* (1426) and *Lactobacillus reuteri* (1428) were grown in deMan, Rogosa and Sharpe (MRS) broth (pH 5.5-6; Difco) under anaerobic conditions (5% H2, 10% CO2, 85% N2, 37°C) to 10⁶ cfu/mL. The cultures were diluted 10x and the matrix plugs were inoculated with 20 µL of 10⁴ cfu/mL under aerobic conditions in the 96 well plate. Each week an inoculated matrix plug was placed in a culture tube containing MRS or Brain Heart Infusion (BHI) broth (pH 7; Difco) and returned to anaerobic conditions to monitor bacterial growth (visual turbidity determined by a trained laboratory technician). This analysis was performed in duplicate. A matrix control consisted of inoculating a culture tube with 20 µL of the 10⁴ cfu/mL.
lactobacillus cultures. Another control was used in which an uninoculated matrix plug was added to a BHI culture tube. Inoculated matrix plugs were stored in the 96 well plate wrapped in parafilm at 4°C for up to a month under aerobic conditions.

Samples were processed for scanning electron microscopy (SEM) by fixing the matrix plugs with 2.5% glutaraldehyde in 0.1M imidazole buffer solution (pH 7.2) for 5 hours, dehydrating in an ethanol series (50%, 80%, 100%), freezing in liquid nitrogen, fracturing with a cold surgical scalpel blade, thawing fractured pieces into absolute ethanol (5-10 minutes), critical point drying from CO₂, and sputter coating with a thin layer of gold. Samples were examined with a Quanta 200 FEI environmental scanning microscope (FEI Co., Inc., Hillsboro, OR) operated in the high vacuum/secondary electron imaging mode.

Results and Discussion

Anaerobic bacterial growth of *L. acidophilus* and *L. reuteri* was observed for all of the inoculated matrices stored for up to a month at 4°C under aerobic conditions (Table I). In all cases there was bacterial growth in the control tubes inoculated with the lactobacilli without matrix and no bacterial growth was observed in BHI control tubes containing uninoculated matrix. Inoculated matrix stored for up to a month at 4°C under aerobic conditions had shrunk to a hard pellet. Yet the matrix was able to preserve the viability of the lactobacilli for a month under these conditions.

Table I. Growth of Lactobacilli in Carbohydrate Matrices

<table>
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<tr>
<th>Time (days)</th>
<th>Alg-Ca</th>
<th>POS-Alg-Ca</th>
<th>MCP-Alg-Ca</th>
<th>FOS-Alg-Ca</th>
<th>Syr-Alg-Ca</th>
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<tr>
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Alg-Ca = Alginic acid-calcium  
POS = Pectic oligosaccharides  
MCP = Modified citrus pectin  
FOS = Fructo-oligosaccharides  
Syr = Inulin  
1426 = *Lactobacillus acidophilus*  
1428 = *Lactobacillus reuteri*  
+ = growth  
+ = no growth
Scanning electron microscopy of the alginic acid-calcium matrix revealed a honeycomb-like structure (Figure 1) very similar to the pectin-calcium matrix structure reported previously (7). This structure included many cavities for growth of lactobacilli (Figure 2). Since alginic acid is not known to have probiotic properties, the MRS culture medium was responsible for this growth of lactobacilli. When pectic oligosaccharides and modified citrus pectin were included in the alginic acid-calcium matrix, the honeycomb-like structure was more compact with smaller internal cavities (Figure 3). The galacturoniac acid content of MCP was higher than that present in POS (5, 6), making MCP potentially more crosslinked by calcium. The surface of the MCP-alginic acid-calcium matrix was more rough and bumpy (Figure 4), due to the calcium crosslinking of both pectin and alginate networks. Lactobacilli were only observed scattered on the surface of the POS- and MCP-alginic acid-calcium matrix (Figure 4) after three days of growth in BHI broth with inoculated matrix plugs stored at 4°C under aerobic conditions for two weeks. The BHI broth does not support as active growth of lactobacilli compared to MRS (which is selective for lactobacilli). When POS- and MCP-alginic acid-calcium matrix plugs, stored at 4°C under aerobic conditions for three weeks or more, were placed in MRS broth under anaerobic conditions, lactobacilli grew so rapidly that the plugs disintegrated in a day of growth. Therefore, these samples were not processed for electron microscopy. When FOS and inulin were used in the alginic acid-calcium matrix, the internal cavities were packed with lactobacilli (Figure 5) after a day of growth in MRS broth. Bacterial growth expanded in the FOS- and inulin-alginic acid-calcium matrices such that the matrix structure began to break (Figure 6) and the plugs eventually fell apart.

Figure 1. General SEM image of the alginic acid-calcium cross-linked matrix plug four days after transfer of the inoculated (Lactobacillus acidophilus) plug to BHI broth and anaerobic conditions. No probiotic was included in this matrix plug and it was not stored at 4°C under aerobic conditions. Scale bar = 0.5 mm.
Figure 2. Growth of Lactobacillus acidophilus (A) and Lactobacillus reuteri (B) in the alginic acid-calcium matrix without a probiotic. Conditions are the same as in Figure 1. Scale bars = 10 μm.

Figure 3. Structure of the alginic acid-calcium matrix when POS (A) and MCP (B) were included. Scale bars = 0.1 mm.
Figure 4. Growth of *L. reuteri* on the POS-alginic acid-calcium matrix (A) and MCP-alginic acid-calcium matrix (B) three days after transfer of an inoculated plug to BHI broth and anaerobic conditions. These inoculated matrix plugs were stored at 4 °C under aerobic conditions for two weeks prior to transfer. Bacterial cells are labeled with arrows. Scale bars = 10 μm.

Figure 5. Growth of *L. acidophilus* in the FOS-alginic acid-calcium matrix (A) and inulin-alginic acid-calcium matrix (B) one day after transfer of an inoculated plug to MRS broth and anaerobic conditions. These inoculated matrix plugs were stored at 4 °C under aerobic conditions for four weeks prior to transfer. Scale bars = 20 μm.
Figure 6. Growth of L. acidophilus in the FOS-alginic acid-calcium matrix (A) and inulin-alginic acid-calcium matrix (B) two days after transfer of an inoculated plug to MRS broth and anaerobic conditions. These inoculated matrix plugs were stored at 4 °C under aerobic conditions for three weeks prior to transfer. Scale bars = 20 μm.

Since bacterial growth was observed even when prebiotics were not included in the alginate acid-calcium matrix, and we did not quantitatively determine the amount of bacterial growth resulting from the different matrices, it is not possible to compare the prebiotic effects of the different oligo-/polysaccharides used in these symbiotics. However, we know that POS did not have a prebiotic index equivalent to FOS until 24 hours of growth in mixed batch fecal cultures (5). Therefore, it is likely that the fructooligosaccharides produced more rapid growth of lactobacilli compared to POS in these symbiotics. In vitro fluorescent in situ hybridization (FISH) assays using 16s rRNA probes demonstrated that POS and FOS produced significant increases in Bifidobacteria and Eubacteria while increases in lactobacilli did not reach significant levels (5). POS produced by enzymatic hydrolysis of commercial pectin supported growth of L. acidophilus as well as Bifidobacteria and prebiotic index values steadily increased during 48 hours in mixed batch fecal culture (4). Therefore, it is anticipated that a more gradual sustained prebiotic effect would be possible using POS compared to FOS in the alginate acid-calcium symbiotics. This would be an advantage for controlled release of probiotic bacteria, and the short-chain fatty acids they produce, into more distal regions of the colon. Future research will examine whether or not these hypotheses are correct.

Plant fibers have a protective effect on probiotic bacteria. Wheat dextrin was an excellent carrier for L. rhamnosus during freeze-drying and in chocolate-coated breakfast cereal (8), oat flour with 20% β-glucan increased the survival of this probiotic in apple juice (3) and apple slices were used to immobilize casein for lactic acid production and milk fermentation (9). We observed that
the viability of lactobacilli probiotics was preserved for a month in the oligo-
poly-saccharide alginic acid-calcium matrices.

Conclusions

The alginic acid-calcium matrix provided an excellent structure for the
growth of lactobacilli. This matrix is compatible with various plant oligo-
and poly-saccharide prebiotics. Through careful selection of the prebiotic, probiotic
bacteria can be delivered to the colon from a symbiotic matrix in a viable,
actively growing state. It may be possible to direct the delivery of actively
growing probiotic bacteria to different regions of the colon based on the
prebiotic selected in the symbiotic matrix.

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References

1. Gibson, G. R.; Roberfroid, M. B. Dietary modulation of the human colonic
microbiota: introducing the concept of prebiotics. J. Nutrit. 1995, 125,
1401-1412.
2. Deardorff, J. Germs that will fight for you. Chicago Tribune
http://www.venturacountystar.com/news/2007/sep/10/germs-that-will-fight-
3. Luchansky, J. B. Use of biotherapeutics to enhance animal well being and
food safety. Proceedings of the 6th International Feed Production
4. Olano-Martin, E.; Gibson, G. R.; Rastall, R. A. Comparison of the in vitro
bifidogenic properties of pectins and pectic-oligosaccharides. J. Appl.
Widmer, W.; Yadav, M. P.; Gibson, G. R.; Rastall, R. A. In vitro
determination of prebiotic properties of oligosaccharides derived from an
2005, 71, 8383-8389.
2006, 20, 859-864.