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GROWTH STUDIES OF PROBIOTIC BACTERIA ON SHORT CHAIN GLUCOMANNAN, A POTENTIAL PREBIOTIC SUBSTRATE

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14. ABSTRACT

This report describes a 3-year study, completed in September 2009 by the Natick Soldier Research, Development and Engineering Center, to investigate the prebiotic potential of glucomannan (GM) oligosaccharides as a novel dietary approach to assist in alleviating gastrointestinal distress in deployed troops. Konjac flour was enzymatically hydrolyzed using cellulase and β-mannanase. A comparison study was done with cellulase produced GM (GM_c) and two commercial prebiotic substrates. All three substrates had similar degree of polymerization (DP) of 2-9. Five probiotic bacteria were evaluated for growth with these substrates, four *Bifidobacteria* (three *B. bifidum* strains and a *B. longum)* and a *Lactobacillus reuteri*. The growth on the GM_c after normalizing for glucose content in the GM_c was due to glucose in the hydrolysate, not the GM_c. β-mannanase produced GM (GM_β) resulted in a DP=2-7 and a much lower glucose content than GM_c (DP=2-3). An expanded list of *Bifidobacteria* and *Lactobacillus* species were evaluated for growth on the GM_β substrate; no growth was evident with the GM_β. However, non-traditional probiotic sporeforming bacteria, *Bacillus subtilis and Bacillus coagulans*, grew very well on GM_β. In addition, *B. subtilis* grown in GM_β secreted an antimicrobial substance not detected in media containing glucose or another commercial prebiotic. It was concluded that low molecular weight GM hydrolysates do not support the growth of *Bifidobacteria* and *Lactobacillus* bacteria; however, it will support the growth of non-traditional probiotic sporeforming bacteria and can enhance their probiotic potential.

15. SUBJECT TERMS

GUT	PREBIOTICS	BIFIDOBACTERIA	DIGESTION(BIOLOGY)
DIET	PROBIOTICS	LACTOBACILLUS	PROBIOTIC ORGANISMS
SPORES	BACTERIOCINS	MILITARY RATIONS	DIETARY SUPPLEMENTS
BACILLUS	KONJAC FLOUR	COLONIES(BIOLOGY)	DIARRHEA PREVENTION
INTESTINES	SPOREFORMERS	GASTROINTESTINAL SYSTEM	

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PREFACE

This report describes a study conducted by the Natick Soldier Research, Development and Engineering Center (NSRDEC) to evaluate novel dietary approaches that may assist in alleviating gastrointestinal distress in deployed troops. The period of performance was from October 2006 to September 2009. The study was performed under project number AH52, 6.1 Basic Research, and program element number PE 611102. The program evaluated one particular carbohydrate prebiotic, glucomannan, in an effort to develop a fundamental understanding of how the prebiotic affects probiotic bacteria.

GROWTH STUDIES OF PROBIOTIC BATERIA ON SHORT CHAIN GLUCOMANNAN, A POTENTIAL PREBIOTIC SUBSTRATE

1. INTRODUCTION

This report describes a 3-year study, begun in October 2006, on prebiotics conducted by the Natick Soldier Research, Development and Engineering Center (NSRDEC). The objective was to investigate the potential for growing probiotic bacteria on a carbohydrate prebiotic substrate in an effort to better understand how the prebiotic affects probiotic bacteria and ultimately introduce dietary practices to deployed troops that will improve the function of their gastrointestinal (GI) tract. The potential prebiotic evaluated was short chain glucomannan (GM). The source of the GM was konjac flour, a high molecular weight polymer of GM derived from the root of the plant *Amorphophallus konjac*. The konjac flour was hydrolyzed by two different enzymes, cellulase and β-mannanase, producing two different short chain hydrolysate products for potentially growing probiotic bacteria.

According to the American College of Gastroenterology, Arlington, Va., more than 95 million Americans experience some kind of digestive problem. GI disorders range from minor ailments such as diarrhea, constipation, and irritable bowel syndrome to more serious illnesses that require hospitalization (Ohr, 2002). These ailments are usually not life threatening, but they will incapacitate an individual for a period of time. GI problems can be an even bigger problem for military personnel, than for most other Americans, due to the stressful conditions they operate under in the field and the strict diet of high density and high caloric food required in the military food system. These factors can affect the immunological system, as well as create GI problems.

There is a need to continually evaluate novel dietary approaches that may assist in alleviating GI distress in deployed troops. According to Sanders (2005a; 2005b), 76% of the 4,348 military volunteers deployed to Iraq and Afghanistan reported at least one diarrhea episode during their deployment, and more than half of them reported multiple episodes. Based on the reduction in job performance, amount of medical care needed, and resulting work lost, the authors concluded that novel research programs designed to decrease the impact of diarrhea are a great need. In a review of multiple infectious disease challenges of military personnel, Zapor and Moran (2005) note that diarrheal illness is a well known threat to military operations and remains problematic. Aronson's review of multiple infections of deployed American military forces included respiratory illness, tuberculosis, Q fever, and gastroenteritis among others (2006). Aronson notes that those personnel in Iraq who tended to experience symptoms of greater severity and longer duration were likely to have multiple episodes and that rates of diarrhea correlated with local food consumption. Burnette and colleagues (2008) used an algorithm to prioritize naturally occurring infectious disease threats to the U.S. Military. Of the 53 diseases of military significance, the global risk severity index (GRSI) ranked bacterial diarrhea as one of the top three infectious diseases.

Prebiotics is a relatively new scientific concept first introduced in 1995 (Gibson et. al., 1995) that could provide some relief from the everyday problems that people have with their GI tract. Much has been done in the area of prebiotics in the scientific and industrial communities. Basically, a prebiotic has to meet two main criteria to be considered a good prebiotic candidate. First, it has to be resistant to digestion and absorption in the GI tract before reaching the large intestine. Second, it must be fermented by host intestinal microbiota and selectively stimulate the growth and/or activity of certain groups of bacteria that are seen as beneficial to human health.

Though there has been great interest in prebiotics, the bulk of the research and development has been done on four prebiotics: fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, and inulin. However, the carbohydrate GM has shown some potential promise in the literature as a prebiotic (Chen et. al., 2005, Al-Ghazzewi et. al., 2007, Abdulmnen et. al., 2008, Connolly et. al., 2010). GM oligosaccharides are found abundantly in roots, tubers, and many plant bulbs and serve as energy storage carbohydrates in plants. The most commonly utilized GM is extracted from the root of the *Amorphophallus konjac* plant. Konjac corms have been grown for centuries in Asia, where they have provided a source of food with very interesting physical characteristics (Al-Ghazzewi et. al., 2007). The konjac GM oligosaccharides are high molecular weight polymers where the molecular weight typically exceeds 1 X 10^6 daltons. The sugars are arranged in blocks of mannose residues interrupted with one or two glucose residues which are β-1,4 linked with typically 1.6:1 mannose to glucose residues within the polysaccharide (Figure 1). It is generally believed that konjac GM is not degraded in the human digestive tract (Matsuura, 1998).

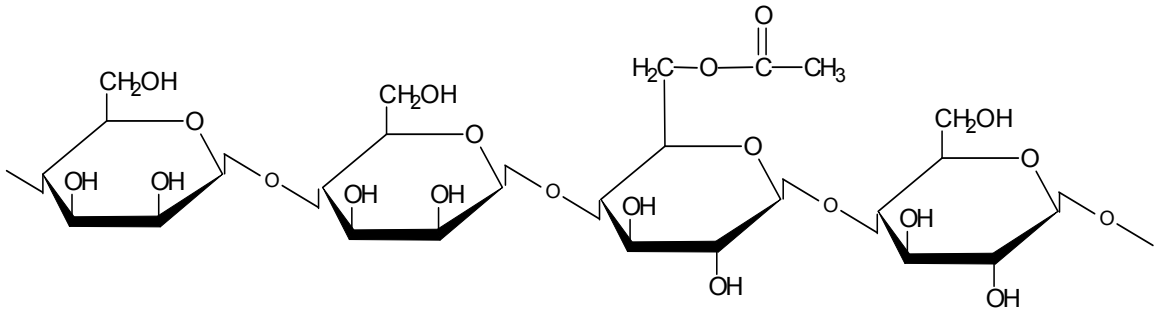

Figure 1. Konjac structure and the basic polymeric unit: GGMMGMMMMMGGM

2. MATERIALS AND METHODS

In this study, cellulase and β-mannanase enzymes were used to hydrolyze konjac flour to produce low molecular weight GM oligosaccharides (GM_c and GM_β, respectively). The structural characteristics and glucose content of both of the GM hydrolysates were characterized by high pressure liquid chromatography (HPLC). Next, they were evaluated for their resistance to digestion and absorption in the GI tract before reaching the large intestine. Three studies were then conducted on their prebiotic potential by growing known probiotic bacteria on them in liquid media and measuring the amount of growth. Most of the probiotic bacteria studied were from the genera *Bifidobacteria* and *Lactobacillus*. Those probiotic bacteria were then grown on two commercial (control) prebiotics, one fructose based and the other glucose based, of similar size (molecular weight), as well as on glucose (positive) and non-substrate (negative) controls. The growth on the various substrate sources for each bacteria was compared.

2.1 Hydrolysis of Konjac

The konjac flour used in the hydrolysis experiments was Propol RS from SunOpta Inc. (Bedford, MA) with a molecular weight of 200,000 – 2,000,000 daltons. The cellulase enzyme used in this study was from *Trichoderma reesei* ATCC 26921 (Sigma, catalogue # C8546), and the β-mannanase enzyme was from *Aspergillus niger* (Megazyme International, Wicklow, Ireland).

The konjac flour was hydrolyzed to short chain hydrolysate products which would be in theory easier to break down and be utilized by the probiotic bacteria. The enzymes were allowed to work for long periods of time at a relatively high temperature to assure full hydrolysis of the original polymer. The buffer used for the hydrolysis of the konjac was a 0.05 M sodium acetate buffer pH 4.5 (Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). A 5% solution of konjac in buffer was used. It was important that the enzyme was added into the buffer solution before the konjac because it swells. If added to the buffer solution before the enzyme, the konjac would congeal and make it impossible to stir the solution.

The amount of cellulase added was 3.75 mg/mL (22.5 units of cellulase/mL) of solution. The 5% konjac solution was shaken at 30 rpm on an Innova 3000 platform shaker (New Brunswick Scientific) at 60 ºC for 3 h. The enzyme was inactivated at 80 ºC for 15 min. The solution was centrifuged at 7500 rpm (SL-250T rotor) on a Sorvall Super T21 (Dupont) centrifuge. The solid was discarded, and the soluble hydrolysate product was freeze dried overnight on a Freezone 6 freeze dry system (Labconco). The total yield of GM short chain oligomers with cellulase was 97.5% GM and glucose.

The konjac flour was hydrolyzed by β-mannanase in a similar way as it was by cellulase with three exceptions. For 5% konjac solution the amount of β-mannanase used was 30 units/mL in acetate buffer, as opposed to 22.5 units of cellulase/mL. The konjac/mannanase solution was stirred at 30 rpm; however, the optimum temperature was 37 ºC for 3 h. The yield of GM short chain oligomers and glucose was only 90%, as opposed to 97.5% for the konjac/cellulase solution.

Cellulase was chosen as an enzyme for this study based on the Al-Ghazzewi et.al. (2007) paper on the potential of enzymatically hydrolyzed konjac GM. It was the only enzyme investigated in that study, which was published early in this NSRDEC project period. In addition to cellulase, NSRDEC's work also investigated konjac flour hydrolysis with β-mannanase as an additional hydrolyzing enzyme for evaluation based mainly on work done in the 1970s characterizing konjac structure by enzyme hydrolysis (Kato et.al. 1970, Shimahara et.al, 1975a, Shimahara et.al. 1975b). In the cited studies, the authors were interested in characterizing the structure of konjac by enzymatic and acid hydrolysis. By producing small chain oligosaccharides with different hydrolysis methods, they could reveal the structural make-up of konjac. The use of β-mannanase was particularly interesting to the NSRDEC team because of the number of different small chain oligosaccharides produced (more than the two produced with cellulase) and the low amount of glucose in final product.

2.2 Glucose Analysis

A glucose (HK) assay kit (Sigma catalogue # GAHK20-IKT) was used to determine the glucose content of the short chain GM produced from cellulase and β-mannanase.

In addition, a glucose oxidase/peroxidase kit was used to determine the free glucose content of the GM_c, GM_β, the two commercial prebiotics, and the unhydrolyzed konjac.

2.3 Evaluation Method to Determine Prebiotic Resistance to Digestion

A good prebiotic candidate has to be resistant to digestion and absorption in the upper GI tract so that the substrate is available in the large intestine, where it provides its benefits. Thus, the commercial prebiotics, konjac hydrolysate products, and unhydrolyzed konjac were evaluated for their resistance to digestion before the in-vitro growth studies were performed. The basic method calls for pepsin digest for protein removal, followed by α-amylase digest, then an amyloglucosidase digest. This method only represents a portion of the digestive enzymes that a prebiotic encounters. There are at least 20 other enzymes and 4 or 5 starch enzymes in the digestive tract.

The method was adapted from Goni et.al. (1996) with minor adjustments. The Goni method was adapted from Berry (1986) with modifications to remove protein to enhance α-amylase accessibility by avoiding starch-protein interactions. Mainly, this step is advisable to better simulate physiological conditions (proteolytic digestive enzymes, acidic pH). This method consists of the following steps:

1. Weigh out 50 mg of dry sample into a 50 mL centrifuge tube. Add 10 mL of KCl-HCl buffer, pH 1.5. Incorporate powder carefully while setting aside 9 mL of solution for Step 3 below.
2. Bring 0.5 mL of solution to 25 mL using de-ionized water, and determine glucose concentration.

3. Add 0.2 mL of a pepsin solution to 9 mL from Step 1 above. Hold for 60 min in a 40 ºC water bath with constant shaking.
4. Cool samples to room temperature, add 9 mL of Tris-maleate buffer, bring to 19 mL, and adjust pH to 6.9. Add 1.0 mL of α-amylase solution, and incubate for 16 h at 37 ºC with constant shaking.
5. Set aside 10 mL for Step 8 below. Centrifuge remaining 10 mL, and save supernatant. Wash pellet with 10 mL water, centrifuge, and save supernatant. Combine supernatants, and determine glucose concentration.
6. To pellet from Step 5, add 3 mL of water and 5 mL 2 M KOH to solubilize resistant starch, mix, and leave for 30 min. Add 5.5 mL of 2 M HCl and 3 mL of 0.4 M sodium acetate buffer, pH 4.75. Add 100 µL of amyloglucosidase, vortex, and leave for 45 min at 60 ºC shaking.
7. Cool to room temperature, dilute to approximately 20-100 µg/mL, and determine glucose concentration.
8. To 10 mL from Step 5 above, add 5 mL 2 M KOH to solubilize resistant starch, mix, and leave for 30 min at room temperature. Add 5.5 mL of 2M HCl and 3 mL of 0.4 M sodium acetate buffer, pH 4.75. Add 100 µL of amyloglucosidase, vortex, and leave for 45 min in a 60 ºC water bath with constant shaking.
9. Cool to room temperature, dilute to approximately 20-100 µg/mL, and determine glucose concentration.

The reagents used in this method are pepsin (Sigma P-7012), porcine pancreatic α-amylase (Sigma A-3176), amyloglucosidase from *Aspergillus niger* (Sigma A-7420), glucose oxidase-peroxidase kit (Sigma G3660), o-dianisidine (Sigma D-2676), and glucose standard (Sigma G-3285).

2.4 In-Vitro Growth Studies

Three growth studies were conducted. The selections of materials and methods used in the second and third studies were shaped by the results from the preceding studies.

First, reinforced clostridium medium (CM) was used with the GM_c, the two commercial prebiotics, and a substrate of 0.24% glucose control for the GM_c to grow five different bacteria: *Lactobacillus reuteri* ATCC 23272, *Bifidobacteria longum* ATCC 55813*, Bifidobacteria bifidum* ATCC 29521, *Bifidobacteria bifidum* ATCC 15696, and *Bifidobacteria bifidum* ATCC 700542. The optical density (OD) of the bacteria was measured after they were grown under anaerobic conditions for 48 h at 37 ºC.

Next, reinforced CM was used with 1% GM_β, a negative (no glucose) control, and a positive (1% glucose) control to grow the same five bacteria and six additional bacteria: *Bifidobacteria animalis* NRRL B-41405, *Bifidobacteria breve* NRRL B-41408, *Bifidobacteria infantis* NRRL B-41661, *Bifidobacteria longum* NRRL B-414409, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* QMB 1474. In addition, minimal medium (MM) was used with the three equivalent sources (only 0.1% GM_β and 0.1% glucose, instead of 0.1%) to grow two non-traditional bacteria: *Aerobacter mannanolyticus* and *Clostridium butyricum*. Each of the 13

bacteria was grown in a separate test tube for the GM_β and each control over an 8 d period, and the three test tubes for each bacterium were compared. To verify that these bacteria were not just slow growers in GM, the tubes were incubated an additional 2 weeks in the anaerobic chamber at 37 ºC. A plus or minus was assigned according to the change in turbidity of the test tubes over the course of the incubation. A minus means no growth (i.e., no change in turbidity). Pluses mean some change in turbidity indicating growth, depending on the number of pluses.

Finally, MM was used with GM_β, the fructose-based commercial prebiotic, and a glucose control at both 0.1% and 0.5% concentrations to grow two sporeforming bacteria: *Bacillus coagulans* ATCC 8083 and *Bacillus subtilis* QMB 1611. The OD of the bacteria was measured at various intervals for 60-80 h under anaerobic conditions at 37 ºC.

2.4.1 Commercial Prebiotics and Probiotic Bacteria Evaluated

Commercial prebiotics used in the study as control substrates were NutraFlora (GTC Nutrition, Golden, Colorado) and VitaSugar IMO powder (BioNeutra, Edmonton, Canada).

A number of probiotic bacteria, as well as sporeformers and other bacteria that had been suggested in the literature as utilizing GM, were obtained for the study. Bacteria obtained from the American Type Culture Collection were *Bifidobacteria bifidum* ATCC 700541, *Bifidobacteria bifidum* ATCC 29521, *Bifidobacteria bifidum* ATCC 15595, *Bifidobacteria longum* ATCC 55813, *Lactobacillus reuteri* ATCC 23272, and *Lactobacillus acidophilus* ATCC 4356. Additional probiotic bacteria obtained from the US Department of Agriculture collection were *Bifidobacteria animalis* NRRL B-41405, *Bifidobacteria bifidum* NRRL B-41410, *Bifidobacteria breve* NRRL B-41408, *Bifidobacteria infantis* NRRL B-41661, and *Bifidobacteria longum* NRRL B-41409. Two sporeforming *Bacillus* bacteria were obtained from Dr. Anthony Sikes of the Combat Feeding Directorate (CFD): *Bacillus subtilis* QMB1611 and *Bacillus coagulans* ATCC 8083. Two other bacteria obtained from Dr. Sikes of CFD were *Aerobacter mannanolyticus* QMB 161 and *Clostridium butyricum* 859.

2.4.2 Media Used in Growth of Bacteria

The GM_c powder and the GM_β powder produced by enzyme hydrolysis, and the two commercial prebiotics were used to grow the probiotic bacteria in reinforced CM or MM Davis Broth, depending on the bacteria of interest.

The reinforced CM was taken from the recipe of DIFCO for reinforced CM. It was used to culture the *Bifidobacteria* and *Lactobacillus* bacteria. One component, glucose, was removed from the original recipe. This was replaced with either 1% or 2 % GM, depending on the particular test, made enzymatically or the commercial prebiotics to determine if bacteria utilized the selected substrates. The recipe consisted of the following components, per L:

- Bacto Tryptone 5.0 g
- Proteose Peptone 5.0 g

- Beef Extract 10.0 g
- Yeast Extract 3.0g
- Sodium Chloride 5.0g
- Soluble Starch 1.0 g
- Cysteine HCl 0.5 g
- Sodium Acetate 3.0 g
- Agar 0.5 g

The MM Davis Broth consisted of the following components, per L:

- Potassium Phosphate (Dibasic) 7.0 g
- Potassium Phosphate (Monobasic) 2.0 g
- Ammonium Sulfate 1.0 g
- Sodium Citrate 0.5 g
- Magnesium Sulfate heptahydrate 0.1 g

The MM was used to grow *Aerobacter*, *Clostridium,* and *Bacillus* bacteria so that no other carbon source would interfere with the growth results. The GM produced enzymatically was substituted for glucose to determine if bacteria utilized the substrate. MM could not be used with bacteria such as *Bifidobacteria* and *Lactobacillus* because they are more fastidious and require other trace elements/nutrients to grow.

3. RESULTS

3.1 HPLC Analysis of Substrate Structures

The β-mannanase used in this study to hydrolyze konjac flour produced more variety of short chain GM oligosaccharides for in-vitro growth studies of probiotic bacteria than the cellulase and far less glucose in the final product than the cellulase. The HPLC characterization revealed two short chain cellulase oligosaccharides with a degree of polymerization (DP) of 2 to 3 and 12-13% glucose. In contrast, HPLC analysis of konjac hydrolyzed by β-mananase produced a small amount of glucose (< 1 %) with six other peaks showing a DP of 2 through 7 (Figure 2). These results conform to the work of Shimahara et.al. (1975b) and Cescutti et.al. (2002). Table 1 provides the structural characteristics of both GM hydrolysates and the two commercial prebiotic substrates (NutraFlora and VitaSugar IMO) that were included as controls.

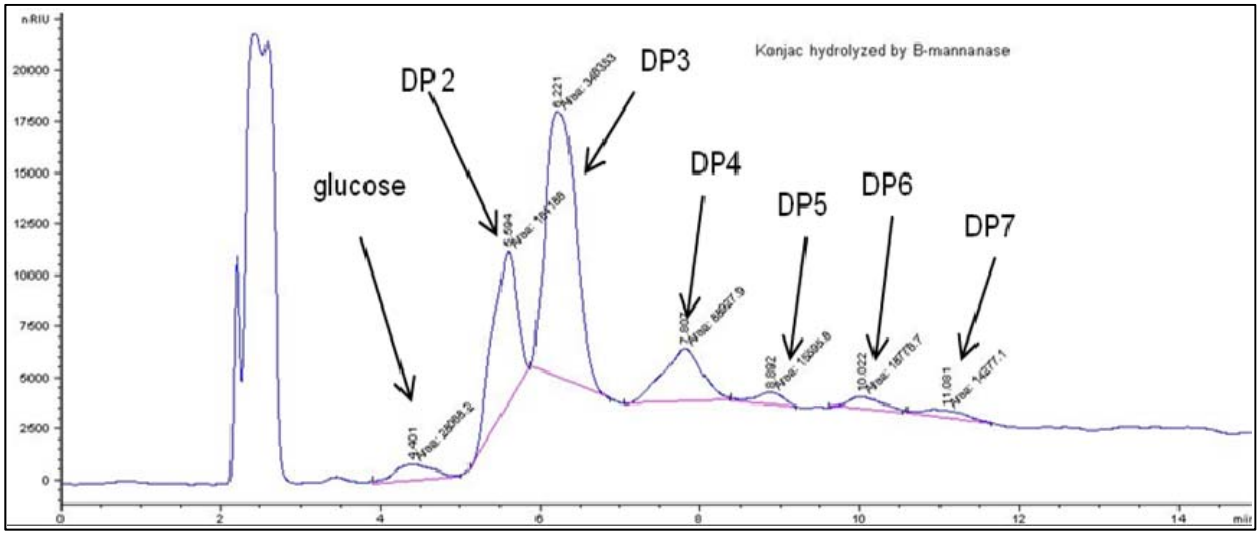

Figure 2. HPLC chromatograph of konjac flour hydrolyzed by β-mannanase

Table 1. Structural comparison of commercial (control) prebiotics and GM hydrolysates

Oligosaccharide	Structure	Degree of Polymerization	Linkage	Glucose Content	Source of Oligosaccharides
Konjac (GM_c)	(Gu)-(Mn)	2-3	β (1,4)	12-13 %	Cellulase hydrolysis (in-house)
Konjac (GM_β)	(Gu)-(Mn)	2-7	β (1,4)	< 1.0 %	β -mannanase hydrolysis (in-house)
NutraFlora (FOS)	(Gu)-(Fr)	2-5	β (1,2)	0.8 %*	Synthetic (commercial)
VitaSugar IMO	(Gu)	2-9	α (1,6)	1.0 %	Liquid starch hydrolysis (commercial)

* Glucose and fructose
Gu = Glucose, Mn = Mannose, Fr = Fructose

Glucose content is an important factor in the in-vitro growth studies of the probiotic bacteria. Ideally a prebiotic would have a low percentage of glucose because the glucose portion of the

substrate will be metabolized before it reaches the large intestine, making that portion of the substrate unavailable for its beneficial effect in the large intestine. Removing glucose is no trivial matter when in a substrate matrix. The low glucose level is a fortunate result of the hydrolysis for in-vitro growth studies and thus gives β-mannanase a significant advantage over cellulase.

3.2 Evaluation of Resistance to Digestion

The large intestine is where the prebiotic should do its work in providing a good substrate source of carbon for the beneficial bacteria of the gut. Therefore, to be a potential prebiotic, a substrate should have a low glucose content (or have it removed) and should resist digestion by enzymes in the human intestinal tract. The results of the evaluation of the GM hydrolysates, the two control prebiotics, and unhydrolyzed konjac flour are presented in Table 2.This study evaluated their resistance to only one enzyme found in the GI tract (α-amylase) and determined the free glucose content of each with a glucose oxidase/peroxidase kit.

Table 2. Evaluation of prebiotic products resistance to digestion

Substrate	Free Glucose	α-Amylase Effect
FOS	0.53 (0.04)	0.03 (0.01)
IMO	1.16 (0.16)	**23.28** (0.14)
Konjac	0.42 (0.04)	0.06 (0.01)
GM_{β}	0.58 (0.04)	0.06 (0.02)
GM_c	**13.04** (0.04)	0.16 (0.00)

Data expressed as mg of glucose as a present of available solid, n = 2 (std. dev)

Two numbers stand out (bold) in the results of the evaluation of prebiotic products for resistance to digestion. First, the high number for IMO under the α-amylase effect indicates that approximately 23% of the substrate is being broken down before it reaches the large intestine. Therefore, nearly one-quarter of the IMO substrate will be metabolized and thus unavailable to perform its beneficial effect, severely limiting its potential as a prebiotic. The second number of interest is the 13% of free glucose in the GM_c. Because the free glucose will be metabolized by the GI tract before reaching the large intestines, more than one-eighth of the GM_c is unavailable for its beneficial effect in the large intestine. Despite the low α-amylase effect on the GM_c, its high glucose content severely limits its potential as a prebiotic unless the glucose can be removed, which is very difficult. Each of the other three substrates (FOS, Konjac, and GM_{β}) showed both high resistance to the amylase effect and low free glucose content, thus potentially making most of the substrate available for metabolism by the beneficial bacteria in the large intestines. These results indicate that FOS, Konjac, and GM_{β} have potential as prebiotics.

3.3 In-Vitro Growth Studies

3.3.1 Five Probotic Bacteria Grown with GM_c

An in-vitro growth study of probiotic bacteria with the selected commercial prebiotics and the GM_c hydrolysate was done using reinforced CM as the base media. Two percent FOS, IMO, and GM_c were substituted for the glucose that is found in CM, producing CMNF, CMIMO, and

$CMGM_c$, respectively. A fourth medium (CMG) was prepared of CM, with the substrate being 0.24% glucose, to act as a control to normalize the medium for the glucose content of the $CMGM_c$. Five probiotic bacteria were selected to grow in these various media. These bacteria were grown under anaerobic conditions for 48 h at 37 ºC.

The results in Figure 3 indicate the two commercial media were selective in supporting the growth of the probiotic bacteria. The IMO prebiotic (CMIMO) supported the growth of three probiotic bacteria, with Bb 70 being the best of the three. The FOS medium (CMNF) supported the growth of two probiotic bacteria, with Bl 55 showing the best growth. It appears that all five prebiotic bacteria grew well on the $CMGM_c$. However, when the CMG is considered, there appears to be little difference in the growth (OD) or pH of the $CMGM_c$ over 48 h. Thus, the results indicate that these five probiotic bacteria did not utilize the GM, but grew on the free glucose in the GM powder.

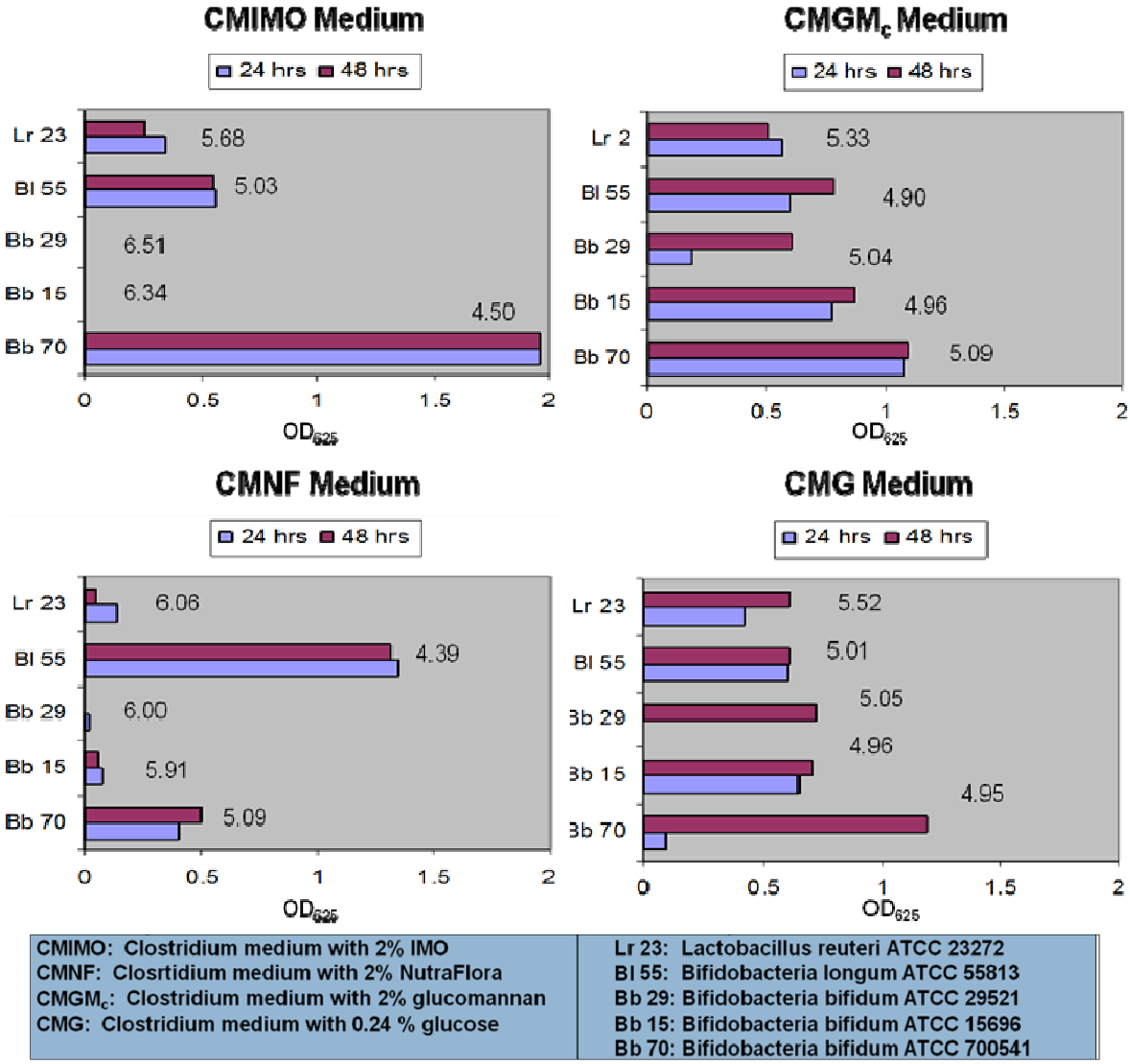

Figure 3. In-vitro growth of bacteria in reinforced CM with FOS (CMNF), IMO (CMIMO), $GM_{,c}$ ($CMGM_c$), and glucose control (CMG)

3.3.2 Thirteen Probotic Bacteria Grown with GM_β

To clarify the issue of whether GM can be utilized by probiotic bacteria in-vitro, given the results of the initial evaluation, additional probiotic bacteria were used, and the GM_β hydrolysate was substituted for GM_c as the carbon source to remove the glucose factor and to increase the variety of short chain GM oligosaccharides. The previously evaluated bacteria and six others that are known to use GM were used in reinforced CM without glucose (CM-) as a negative control, reinforced CM with 1% glucose (CMG) as a positive control, and reinforced CM with 1% GM_β ($CMGM_\beta$). In addition, two non-traditional bacteria were grown in MM with similar carbon sources: no glucose (MM-), 0.1% glucose (MMG), and 0.1% GM_β ($MMGM_\beta$). An MM was used so no other carbon source would interfere with the growth results. This medium was not used with the *Bifidobacteria* or *Lactobacillus* bacteria (the other 11 bacteria evaluated) because they are more fastidious and require trace minerals and other cofactors to grow. The positive and negative control tubes for each bacterium were compared to the test tube containing the GM_β as the substrate over an 8 d period. To verify that these bacteria were not just slow growers in GM, the tubes were incubated an additional 2 weeks at 37 °C in the anaerobic chamber.

Figure 4 shows the results of the study with the various bacteria evaluated based on a qualitative plus/minus system for growth in the various media according to the change in turbidity of the test tubes over the course of the incubation.

Bacteria	Extended Incubation (3 weeks)		
	CM-	CMG	CMGMβ
Bifidobacteria longum ATCC 55813	+	++++	+
Bifidobacteria bifidum ATCC 29521	+	++++	+
Bifidobacteria bifidum ATCC 15696	+	++++	+
Bifidobacteria bifidum ATCC 700541	+	++++	++ (8 Days)
Bifidobacteria animalis NRRL B-41405	+	++++	++ (8 Days)
Bifidobacteria breve NRRL B-41408	++	++++	++
Bifidobacteria infantis NRRL B-41661	+	++++	+
Bifidobacteria longum NRRL B-41409	+	++++	+
Lactobacillus reuteri ATCC 23272	+	++++	+
Lactobacillus acidophilus ATCC 4356	+	++++	+
Lactobacillus casei QMB 1474	+	++++	++ (8 days)
	MM-	MMG	MMGMβ
Aerobacter mannanolyticus QM B1612	-	++++	++++
Clostridium butyricum 859	-	-	-

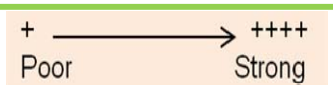

Figure 4. In-vitro growth of bacteria in reinforced CM and MM of no substrate (negative) control (CM- and MM-), glucose (positive) control (CMG and MMG), and GM_β ($MMGM_\beta$ and $CMGM_\beta$)

The highest rating for the 11 traditional bacteria in reinforced CM was for the CMG tubes (the positive control set of test tubes with glucose as substrate); they all reached a plus four. The CM- medium (the negative control, no glucose) indicated some growth, but a minimal (poor) amount. All of the bacteria in the CM- tubes showed only a plus one, except *Bidobacteria breve*

(a plus two). This poor growth was expected because there are a few components in the medium that can be metabolized by the bacteria. There was only a slight increase in growth with the $CMGM_{\beta}$ (one more plus) compared with the CM- in only three species: *Bifidobacteria bifidum* ATCC 700541, *Bifidobacteria animalis* NRRL B41405, and *Lactobacillus casei* QMB 1474. This slight increase in turbidity was seen only during the initial 8 d of incubation. No additional change was indicated over the next 2 weeks in those tubes. At best, the growth with GM_{β} was minimal for these three species. The growth with GM_{β} in the other species using CM was the same as that with the CM-.

However, It was found that *Aerobacter mannanolyticus*, one of the non-traditional bacteria, can grow quite well on the GM_{β}, as well as on the glucose control. Four pluses were recorded for both the $MMGM_{\beta}$ and the MMG. The *Clostridium butyricum*, the other non-traditional bacteria, did not grow on the positive control or on the $MMGM_{\beta}$. These two bacteria were included in this evaluation because of the lack of growth of the traditional bacteria such as *Lactobacillus* and *Bifidobacteria* species and because studies were found in the literature that suggest that enzymes in the feces of human subjects broke down konjac GM associated with *Aerobacter mannanolyticus* and *Clostridium butyricum* bacteria (Innami, 1961; Nakajama and Matsuura, 1997; Matsuura, 1998).

3.3.3 Two Sporeforming Probotic Bacteria Grown with GM_{β}

With the lack of growth of the traditional probiotic bacteria such as *Lactobacillus* and *Bifidobacteria* species and the success of growing *Aerobacter mannanolyticus* on GM_{β} in MM, , two more non-traditional species (sporeformers) were evaluated for their growth on short chain GM_{β} in MM: *Bacillus coagulans* ATCC 8083 and *Bacillus subtilis* QMB 1611. Three substrates were used in the MM: glucose (MMG), the FOS-based prebiotic NutraFlora (MMNF), and GM_{β} ($MMGM_{\beta}$). Two different concentrations of each substrate were analyzed: 0.1% and 0.5%.

Sporeforming bacteria were chosen because they would survive in the long-term storage requirements of military rations: 6 months at 100 ºF or 3 years at 80 ºF. These requirements are difficult to meet for typical vegetative cells, but for spores these conditions would be of no consequence due to spore resistance properties to environmental extremes. In the literature, sporeformers have received interest as probiotic bacteria (Hong et.al., 2005; Sanders et.al., 2003; Duc et.al., 2004).

As shown in Figure 5, at the low concentration of substrate the $MMGM_{\beta}$ supports a faster growth rate than the MMG for *B. subtilis,* incubated anerobically for 60-80 h at 37 °C. Similar results obtained with *B.coagulans*, though the data are not shown. The doubling time for *B. subtilis* in $MMGM_{\beta}$ was 1 h and 40 min while in MMG the doubling time was 2 h. The MMNF supported growth; however, the graph represents diauxic growth in which the sucrose in the substrate is used first by *B. subtilis* and then the FOS. A personal communication with Dr. Robert Hutkins, University of Nebraska, Lincoln, NE, confirmed that this can occur with NutraFlora with certain bacteria.

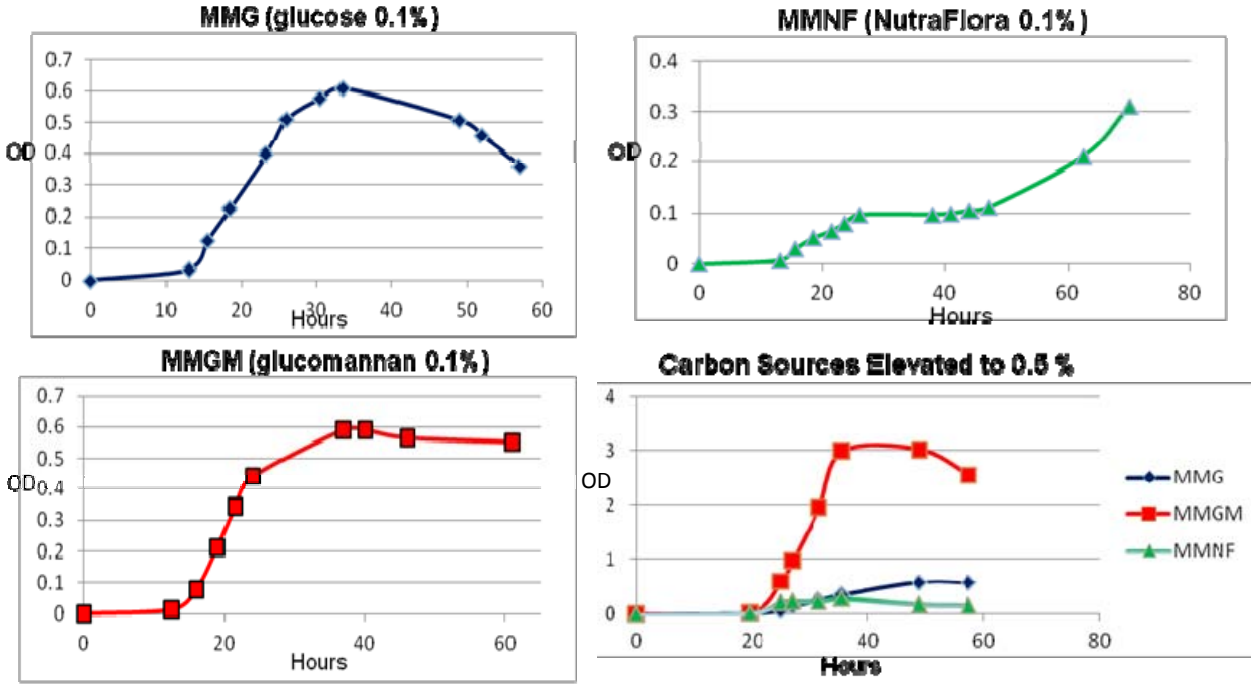

Figure 5. In-vitro growth study of *Bacillus subtilis* QMB 1611 in MM with glucose control (MMG), FOS (MMNF), and GM_{β} ($MMGM_{\beta}$)

The use of a higher concentration of substrate produced a large difference in OD with the $MMGM_{\beta}$ compared with the MMG and MMNF. This was due to a pigment produced by *B. subtilis* at the higher concentration of GM. A viable cell growth curve of *B. subitilis* was done for each medium to determine the colony forming units (CFUs) in each, which indicated that all three media supported good growth at the higher concentration of substrate (1×10^8 bacteria/mL). This confirmed that the higher OD was due to the pigment produced in the $MMGM_{\beta}$. In addition, the supernatant of all three media were analyzed for antimicrobial activity due to the pigment seen in the $MMGM_{\beta}$. It was found that the *B. subtilis* produced an antimicrobial in the $MMGM_{\beta}$, but not in MMG or MMNF. The antimicrobial showed a narrow range of activity only against *Bacillus megaterium* of the bacteria tested, indicated by a zone of clearing around wells containing the supernatant of the medium. It is believed this antimicrobial was a bacteriocin because when heated at elevated temperatures (80 ºC) the antimicrobial still exhibited activity. An electrophoresis gel run of the culture medium indicated it to be a low molecular weight peptide (2500-3300 MW).

4. DISCUSSION

The results indicate that low molecular weight GM does not support the growth of *Bifidobacteria* or *Lactobacillus* species evaluated in this study. Two hydrolyzed GM products were produced from konjac using the enzymes cellulase and β-mannanase. Both GM products were low molecular weight. At first it appeared GM_c supported growth of all the probiotic bacteria; however, once normalized for glucose content in the GM_c there was no growth due to the GM. There was an indication of some growth occurring with the β-mannanase produced GM which had little glucose and additional short chain oligosaccharides compared to the GM_c. By extending the incubation time to 8 days, three probiotic bacteria showed growth in the modified reinforced CM with GM_β. They were *B. bifidum* ATCC 700541*, B. animalis* NRRL B-41405, and *L. casei* QMB 1474. However, the growth was poor at best. The two commercial products, NutraFlora and VitaSugar, appear to selectively support growth of probiotic bacteria. This is not surprising because a prebiotic will not necessarily support the growth of all probiotic bacteria.

These results did not support some of the reported data in the literature in which GM was considered a potential prebiotic substrate. Matsuura (1998) has shown that the feces of human subjects fed konjac contained enzymes that could degrade konjac. These enzymes were traced to human intestinal bacteria which produced short chain fatty acids (SCFAs) from the degraded konjac. In an in-vivo study (Chen et. al., 2005) with mice, the konjac was acid hydrolyzed producing hydrolysates of an average DP of 12. It was determined that the hydrolyzed GM had greater prebiotic effect on the intestinal flora of the mice than the konjac. In the study by Al-Ghazzewi et. al., (2007), enzyme hydrolyzed konjac, with a DP of 10 to 70, was found to stimulate the growth of a number of *Lactobacillus* and *Bifidobacteria* bacteria. Elamir et. al. (2008) indicated that in feeding mice the konjac GM hydrolysates promoted the growth of anaerobes and *Lactobacilli* while reducing the population of *Clostridium perfringens* and *Esherichia coli*. Connolly et. al. (2010), in an in-vitro study in which konjac GM hydrolysate was studied in batch culture inoculated with human feces, indicated an increase in *Bifidobacteria* and *Lactobacillus* populations due to the GM.

In producing and characterizing two different short chain GM products enzymatically from konjac flour, GM_c, and GM_β have proved to be poor substrates in pure culture in-vitro growth experiments for traditional probiotic bacteria, *Lactobacillus* and *Bifidobacteria*. Though this may be true, this does not rule out GM as potential prebiotic. The various studies in the previous paragraph show the potential of GM. However, all of the studies were either done in in-vivo, where they did not account for the glucose in the hydrolysate, or they were in mixed cultures or combinations of these factors that can confound the results of which bacteria are utilizing the GM directly. It may well be that the traditional probiotic bacteria can only utilize the GM once another genera has hydrolysed the substrate. It has been reported previously and shown by this study that konjac and GM are resistant to enzyme digestion through the GI tract, so the GM can make it to the large intestine and potentially be used by the microflora present in the gut.

In this study, non-traditional probiotic bacteria have also been looked at that can utilize GM directly in pure cultures. *Bacillus*, a sporeforming bacterium, has been studied and

commercialized as a probiotic bacterium (Ganeden, Mayfield Heights, OH). In evaluating *Bacillus* it was determined that both *Bacillus subtilis* and *Bacillus coagulans* grew very well with GM_{β} as the sole carbon source in MM. In determining the doubling time of *B. subtilis* with GM_{β} verses glucose, the doubling time was shorter with the GM_{β}. This indicated GM was a better carbon source than glucose for growth. This is interesting because a sporeformer such as *Bacillus* could easily survive the storage requirements of military rations. There is still skepticism in the scientific community about whether sporeformers such as *Bacillus* can provide any benefit to the individual even though they have been commercialized. Clinical studies and proof of definitive beneficial effects have been lacking on the bacteria. Also interesting, there was diauxic growth on the commercial prebiotic substrate, NutraFlora, in MM. *B. subtilis* exhibited a distinct preference for the 3% sucrose contained in the NutraFlora over the FOS. In addition, at a higher concentration of GM, a pigment was produced based on the increase in OD of the culture versus the other two substrates, glucose and NutraFlora. Further analysis has shown that an antimicrobial is being produced in the GM medium by *Bacillus subtilis* not detected in the other medium containing NutraFlora or glucose. From the heat stability of the antimicrobial and its low molecular weight, it is believed to be a bacteriocin. Its activity is narrow in range, only exhibiting activity against *Bacillus megaterium*.

5. CONCLUSIONS

Konjac flour can be hydrolyzed with enzymes to form a soluble short chain GM powder. Using cellulase and β-mannanase to hydrolyze the konjac, a degree of polymerization can be produced of 2-3 and 2-7 GM oligosaccharides, respectively. These short chain GMs in pure culture in-vitro growth studies will not support the growth of traditional probiotic bacteria *Lactobacillus* and *Bifidobacteria*. However, the GM still has potential as a prebiotic because it does survive the effect of digestive enzymes in the GI tract and can be utilized in the large intestine by other bacteria. Non-traditional probiotic sporeforming bacteria such as *Bacillus subtilis* and *Bacillus coagulans* can efficiently grow on GM. In addition, an antimicrobial substance is produced by *B. subtilis* in the GM medium which is not seen in media with glucose or FOS. It is believed to be a bactereriocin, probably subtilin.

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