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4 **Protective effects of natural and partially degraded konjac glucomannan on**

5 **Bifidobacteria against antibiotic damage**

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Abstract

This study was to evaluate the protective effects of a dietary fiber, konjac glucomannan (KGM) from the plant tuber of *Amorphohallus konjac* on *Bifidobacteria* against antibiotic damage. KGM (~8.8×10^8 Da) was partially degraded with high-intensity ultrasound to KGM-US (~1.8×10^6 Da) and then hydrolyzed with trifluoroacetic acid (TFA) to KGM-AH (1369 Da). KGM-US (at 5 g/l) showed the most significant protective effect on most bifidobacterial strains against penicillin and streptomycin inhibition, increasing the minimal inhibitory and bactericidal concentration (MIC and MBC) dramatically, and KGM also showed significant effects on enhancing the MBC of enrofloxacin, penicillin, tetracycline and streptomycin. In addition, the adsorbance ability and biofilm improving effects of KGM and degraded KGM products may partially contributed to the protective effects. The results suggested that KGM and ultrasound treated KGM have protective effects for the human gut probiotic bacteria against the damage caused by specific antibiotics.

Keywords: *Bifidobacteria*; Antibiotics; Konjac glucomannan; Partial degradation; Prebiotic fiber

1. Introduction

Antibiotics had been regarded as the most successful drugs for a long time after the discovery of penicillin in the World War II because of their high efficacy for treatment of infectious diseases and for saving billions of lives (Modi, Collins, & Relman, 2014). With the rapid advancement and expansion of poultry farming and aquaculture in the postwar years, antibiotics have been increasingly used as feed additives for the prevention of diseases in animals and for the promotion of animal growth (Blaser, 2016; Zhao, Dong, & Wang, 2010). However, the excessive use of antibiotics in the poultry and aquafarming processes in recent

42 decades has imposed a health threat worldwide owing to the development of antimicrobial
43 resistance as well as the many side effects of antibiotics on human health. Some of the
44 unabsorbed antibiotics in the upper gut which enter the large intestine may disrupt the gut
45 microbial balance by inhibiting the beneficial bacteria, increasing the colonization of resistant
46 microbes and pathogenic organisms. The imbalanced gut microbiota can lead to gut
47 inflammatory diseases and metabolism disorders (Keeney, Yurist-Doutsch, Arrieta, & Finlay,
48 2014). *Bifidobacteria*, which represent an important group of beneficial probiotic bacteria in
49 human gut microbiota, were found to suffer a significant loss after antibiotic treatment
50 (Dethlefsen, Huse, Sogin, & Relman, 2008).
51
52 Penicillin, enrofloxacin, tetracycline and streptomycin are among the most widely used
53 antibiotics in veterinary medicine and animal feed (Schwarz, 2001; Sumano, Gutierrez, &
54 Zamora, 2003; Voldrich, 1965). Their antimicrobial actions are based on different mechanisms.
55 Penicillin breaks the bacterial cell walls indirectly by targeting on the peptidoglycans of
56 bacteria, and is more effective against the Gram positive bacteria (Winstanley & Hastings,
57 1989). Enrofloxacin, an approved veterinary medicine by the US Food and Drug
58 Administration (FDA), kills bacteria by targeting on the DNA gyrase (Trouchon & Lefebvre,
59 2016). Tetracycline and streptomycin mainly prevent bacterial protein synthesis by inhibiting
60 the combination of aminoacyl tRNA with bacterial ribosome (Chopra & Roberts, 2001;
61 Igarashi, Ishitsuka, & Kaji, 1969).
62
63 Natural polysaccharides extracted from plants and other sources have various bioactivities,
64 such as antitumor and immunomodulation (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007;
65 Yan, Wang, Li, & Wu, 2011), and antioxidant (Ferreira et al., 2015; Huang, Siu, Wang, Cheung,
66 & Wu, 2013; Yue, Ye, Zhou, Sun, & Lin, 2013). As many of the bioactive natural

67 polysaccharides are non-starch and non-digestible, one of their primary sites of action may be
68 in the large intestine on the gut bacteria (Ramberg, Nelson, & Sinnott, 2010; Singdevsachan et
69 al., 2016). Besides the nutritional and biological functions, natural polysaccharides can affect
70 the gut bacteria with their physicochemical properties such as the thickening and gelling effects
71 in aqueous media. The exopolysaccharides or extracellular polymeric substances (EPS) of
72 lactic acid bacteria and other microorganisms may have a protective function for the bacteria,
73 such as the resistance to antimicrobials with the formation of a biofilm barrier to the diffusion
74 and uptake of antimicrobials (Mah & O'Toole, 2001). Mushroom polysaccharides enhanced
75 the survival rate of probiotic bacteria in yogurts during cold storage and improved the tolerance
76 in simulated gastric and bile juices (Chou, Sheih, & Fang, 2013). Nonetheless, only a few
77 studies have been documented on the protective effects of natural polysaccharides on probiotic
78 gut bacteria against antibiotics.

79

80 Konjac glucomannan (KGM) isolated from the tuber of plant *Amorphophallus konjac* C. Koch
81 is commonly used as a gelling and thickening agent in liquid foods and also as an edible film
82 coating of food and pharmaceutical products (Herranz, Borderias, Solas, & Tovar, 2012; Xu,
83 Li, Kennedy, Xie, & Huang, 2007). Recently, KGM has been increasingly used as a dietary
84 fiber in functional foods for improving gut health, lowering blood sugar and cholesterol, the
85 risk of type II diabetes and obesity (Behera & Ray, 2016; Tester & Al-Ghazzewi, 2013, 2016;
86 Zhang, Xie, & Gan, 2005). Native and enzyme-hydrolyzed KGM products have been evaluated
87 as prebiotic substrate for the growth of lactobacilli and bifidobacteria (Al-Ghazzewi, Khanna,
88 Tester, & Piggott, 2007; Al-Ghazzewi & Tester, 2012; Yang et al., 2017) and other probiotic
89 bacteria of human or animal gut microbiota (Connolly, Lovegrove, & Tuohy, 2010; Harmayani,
90 Aprilia, & Marsono, 2014). To the best of our knowledge, however, no previous studies have

91 assessed the protective effects of KGM on bifidobacteria or any other probiotic bacteria against
92 antibiotics.
93
94 This study was to evaluate the protective effects of natural and partially hydrolyzed KGM on
95 *Bifidobacteria* against the inhibition of antibiotics and to investigate the possible mechanisms.
96 The natural KGM was first treated by high-intensity ultrasound (US) to attain partially
97 degraded KGM with relatively high molecular weights. The US-degraded KGM was further
98 degraded to much lower molecular weight with trifluoroacetic acid (TFA). The potential
99 protective effects of various KGM fractions were assessed on five important bifidobacterial
100 species against four representative antibiotics used in medicine and farming, penicillin,
101 tetracycline, enrofloxacin and streptomycin. Two well-known prebiotic carbohydrates, inulin
102 and galactooligosaccharide (GOS) were used as references and tested together with KGM
103 fractions. The possible formation of biofilms on solid surfaces and the absorption of antibiotics
104 to KGM were analyzed.
105
106 **2. Materials and methods**
107 **2.1 Bacterial strains and culture conditions**
108 Five strains of *Bifidoacteria* were used in the present study (Table 1), which were generously
109 donated by Biostime Ltd. The bacterial strains were stored in 15% (v/v) glycerol tubes at -
110 80 °C. The bacteria were cultured in Reinforced Clostridium Medium (RCM) (Guangdong
111 Huankai Bio-Technology Co., Ltd., Guangzhou, China). The RCM medium was composed of
112 5 g/l glucose, 10 g/l beef extract, 10 g/l peptone, 3 g/l yeast extract, 1 g/l soluble starch, 0.5 g/l
113 cysteine HCl, 5 g/l sodium chloride, 3 g/l sodium acetate and 0.5 g/l agar for RCM broth or 15
114 g/l for RCM agar with a final pH of 6.8 ± 0.2 (unadjusted). The culture media were sterilized
115 at 121 °C for 20 min. Prior to the culture experiments, the bacterial strains taken from the

116 storage were cultured on RCM agar solid medium for 48 h. A single colony spot was picked
117 out from the solid culture and inoculated into 5 ml of RCM broth liquid medium in a 10 ml
118 centrifuge tube, followed by shaking incubation at 200 rpm for 24 h. The final bacterial
119 suspension was inoculated at 1% (v/v) into the RCM broth under the same conditions as for
120 the culture experiments. The closure of the centrifuge tube was punctured to ascertain
121 anaerobic atmosphere in the ullage of the tube. All the bacterial cultures were maintained at 37
122 °C under anaerobic condition in air-tight jars with anaerobic gas generating sachets
123 (AnaeroGen TM, Thermo Scientific Oxoid, USA) or (Mitsubishi Gas Chemical Co., Inc.,
124 Tokyo, Japan) (Tanner et al., 2014).

125

126 **Table 1** Five strains of *Bifdobacterium* used in this study.

Microorganism	Strain code	Origin
B. adolescentis	CICC[a] 6070	Intestine of adult
B.bifidum	CICC 6071	Infant feces
B. breve	CICC 6079	Intestine of infant
B.infantis	CICC 6069	Intestine of infant
B.longum	CICC 6186	Intestine of adult

127 [a] CICC: China Center of Industrial Culture Collection (Beijing, China)

128

129 **2.2 Preparation of ultrasound- and acid-degraded KGM**

130 Konjac glucomannan (KGM) was provided by Hubei Konson Konjac Gum Co., Ltd. (Ezhou,
131 Hubei, China). KGM was dissolved in distilled water at 10 g/l and 150 ml of the KGM solution
132 was added to a centrifugal bottle for ultrasonic degradation. Ultrasonic degradation of KGM
133 was carried out as described previously (Li, Li, Geng, Song, & Wu, 2017) with a VCX 750
134 processor (Sonics and Materials Inc., Newton, USA) with a fixed frequency of 20 kHz and a
135 maximum output power of 750 W. A probe horn with a tip diameter of 13 mm was used and
136 the sample was irradiated at a fixed power level of 80% amplitude for 30 min, yielding US-

137 degraded fraction KGM-US. The KGM-US (0.15 g) was treated with 60 ml of 2 M
138 trifluoroacetic acid (TFA) at 70 °C for 4 h, yielding acid-hydrolyzed KGM fraction KGM-AH.
139 After the US and acid treatment, the KGM solutions were evaporated to dryness with a rotary
140 evaporator under vacuum at 40 °C and then washed by methanol. Finally, the degraded KGM
141 samples were redissolved in 10 ml DI water and freeze dried, and stored in a desiccator at room
142 temperature before use.

143

144 **2.3 Measurement of intrinsic viscosity and molecular weight**

145 The intrinsic viscosity of KGM and degraded products was determined as described previously
146 (Yan et al., 2009). The KGM samples were dissolved with distilled water overnight under
147 constant stirring. The solution was diluted with water in series and filtered through a Watman
148 No. 1 paper and the viscosity was measured with an Ubbelohde viscometer (0.5-0.6 mm
149 capillary diameter) at 25 ± 0.1°C. The intrinsic viscosity [η] was derived from the following
150 equations,

151 $\eta_{sp}=(\eta_{sample}-\eta_{ref})/\eta_{ref}=(t_{sample}-t_{ref})/t_{ref}$ (Eq.1)

152 $\eta_{red}=\eta_{sp}/C=[\eta]+k'[\eta]^2C$ (Eq.2)

153 where η_{sp} is the specific viscosity and η_{red} the reduced viscosity and η_{ref} the viscosity of
154 reference (distilled water), C the sample concentration, and k a constant related to the polymer
155 solution.

156

157 Molecular weight (MW) of KGM and other poly- and oligosaccharide samples was measured
158 by a high-pressure gel permeation chromatography (HPGPC) instrument equipped with a
159 Waters 1515 isocratic pump and a 2414 refractive index detector (Waters Co, Milford, MA,
160 USA) as described previously (Huang et al., 2013). A series of three columns was used
161 including Waters Ultrahydrogel 120, 250 and 2000 (7.8 × 300 mm) and the column temperature

162 was 50 °C. The mobile phase was Milli-Q water at a flow rate of 0.6 ml/min. All samples were
163 dissolved in distilled water (0.2 mg/ml for KGM, 1 mg/ml for KGM-US, and 3 mg/ml for
164 KGM-AH) and centrifuged at 6000 rpm for 15 min. The supernatant was collected and filtered
165 through 0.45 μM membrane before the injection. Dextran MW standards 1, 5, 12, 25, 50, 80,
166 270, 410 and 670 kDa were used to obtain the calibration curve.

167

168 **2.4 Preparation of poly- and oligo-saccharide solutions for bacterial cultures**

169 For investigation of the effects of KGM and its degraded products on the antibiotic-treated
170 bifidonacteria, the KGM samples were added to the bacterial medium RCM at three different
171 concentrations (0.5, 2 and 5 g/l). KGM and other poly- and oligo-saccharides were all dissolved
172 in distilled water at the desired final concentrations by stirring for overnight, and then 38 g/l of
173 RCM powder was mixed with each sample solution. The RCM medium containing the KGM
174 and other poly- and oligo-saccharides was sterilized by autoclaving at 121°C for 20 min.

175

176 Putative prebiotic carbohydrate fibers were tested as references for comparison with the KGM
177 fractions including galactooligosaccharides (GOS) and inulin. GOS with purity of 80% was
178 obtained from New Francisco Biotechnology Co., Ltd. (Yunfu, China) and inulin (from dahlia
179 tubers, DP ≈ 36) from Sigma (St. Louis, MO, USA). The solutions were prepared in the same
180 way as for KGM.

181

182 According to the guidelines from the Institute of Medicine, American Heart Association and
183 Chinese Nutrition Society, the recommended intake of dietary fiber for an adult is 25 g to 38
184 g/day or 14 g/1,000 kcal/day while the mean intake was slightly more than 15 g/day (King,
185 Mainous, & Lambourne, 2012). If an adult takes 10 g of carbohydrate fibers per day as dietary
186 fiber supplement, the concentration is about 5 g/l in a total intestinal volume of 2 l (Parkar et

187 al., 2010). Therefore, the concentration of 5 g/l was chosen in the experiments for evaluating
188 the protective effects of KGM and other carbohydrate fibers.
189

190 **2.5 Preparation of antibiotic solutions**

191 Four of the most common antibiotics used in human and animal care were chosen for this study.
192 Enrofloxacin and streptomycin sulfate are two antibiotics commonly applied in livestock
193 husbandry and fishery, while penicillin G and tetracycline hydrochloride are widely used in
194 human and animal medicine. The four were all purchased from Guangzhou XiangBo Bio-
195 Technology Co., Ltd. (Guangdong, China). Antibiotic solutions were freshly prepared in the
196 culture medium at a concentration of 2.5 mg/ml.
197

198 **2.6 Determination of minimum inhibitory and bactericidal concentrations**

199 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)
200 were used to represent the sensitivity of bifidobacteria to antibiotics. MIC was defined as the
201 lowest concentration required for complete inhibition of the bacterial growth (Karakoc &
202 Gerceker, 2001), and MBC as the lowest concentration for killing 99.9% of the initial inoculum
203 (Standards, 1991). MIC and MBC were determined by microtiter plate assays (Cleusix,
204 Lacroix, Vollenweider, Duboux, & Le Blay, 2007). The antibiotics were dissolved in distilled
205 water at 2.048 mg/ml. A serial two-fold dilution of the antibiotic solution was prepared and the
206 diluted solution was transferred at 100 μl aliquots into a 96-well polystyrene microtiter plate
207 (SPL Lifesciences Inc., Pocheon, Korea) containing 100 μl of RCM broth per well. The
208 bifidobacteria were cultured to mid-log phase (16-18 h) in RCM broth as described above. The
209 optical density (OD) of bacterial suspension was adjusted to 0.1 with fresh RCM broth using a
210 Ledetect microtiter plate reader (Labexim, Lengau, Austria) at 600 nm (Mota-Meira, LaPointe,
211 Lacroix, & Lavoie, 2000). Then the standardized bacterial suspension was inoculated at 100 μl

212 into each well, the microtiter plates were incubated anaerobically at 37°C for 48 h and the
213 OD_{600} was recorded. A control inoculated with the tested culture in RCM and a blank
214 containing only RCM were included on each microtiter plate. The first well with OD value
215 equal to the control was taken as MIC. For the MBC assay, 20 µl was withdrawn from the first
216 well showing no visible growth on the RCM agar and the lowest concentration with no colony
217 appearing on the RCM agar was taken as MBC (Cleusix et al., 2007). The microtiter plate assay
218 was performed in four replicates for each antibiotic–bacterium combination and the median
219 MIC or MBC values were recorded as the result.
220

221 **2.7 Test of KGM on bifidobacterial growth**

222 For examination of their effects on the growth of bifidobacteria, KGM, degraded KGM and
223 prebiotic references were added to the RCM medium at 5 g/l final concentration, and then
224 subjected to serial 2-fold dilution from 5 to 0.0782 g/l. The liquid medium was dispended into
225 a 96-well microtiter plate at 200 µl per well, followed by inoculation of the *Bifidobacteria*
226 (4×10^5 colony forming units in total volume) and incubation for 48 h at 37 °C in anaerobic
227 atmosphere. RCM inoculated with bacteria was included as the control and RCM with KGM
228 and prebiotic but no bacteria as the blank. The bacterial concentration was determined by
229 measurement of OD at 600 nm and the treatment effect was represented by (OD_{test} -
230 OD_{blank})/$OD_{control}\times100\%$.
231

232 **2.8 Detection of biofilm formation of *Bifidobacteria***

233 The formation of biofilm as a possible mechanism for the protective effect of KGM against
234 antibiotic damage was detected by modified methods from literature (Stepanovic, Vukovic,
235 Dakic, Savic, & Svabic-Vlahovic, 2000) on bacterial adhesion to surfaces in culture tubes and
236 microplates. In the tube test, a bacterial strain cultured on RCM agar plates was inoculated into

237 glass tubes (13×100 mm) filled with 2.6ml of RCM broth containing 5 g/l KGM or KGM-US.
238 The broth was mixed by pipetting gently and repeatedly and 0.6 ml of the broth was removed
239 from each tube for the microtiter plate test. The tubes containing RCM supplemented with or
240 without KGM and KGM-US (5 g/l) were included in the test as negative control. After
241 incubation anaerobically at 37 °C for 48 h, the liquid was removed from the tubes with a pipette,
242 followed by addition of 2 ml of 0.25% safranin solution into each tube for staining. After
243 removal of the liquid with a pipette, the tubes were placed upside down at room temperature
244 overnight. The amount of bacterial adhered on the inner tube wall was compared by visible
245 observation and recorded as absent (0), weak (+), moderate (++), or strong (+++).
246
247 In the microtiter plate test, 200 μl of bacterial suspension from above tube test was filled in
248 each of three wells of a 96-well microtiter plate (for suspension culture) and the wells filled
249 with 200 μl of RCM but no polysaccharides were included as the control. The covered plates
250 were incubated anaerobically at 37°C for 48 h. After removal of the liquid content, each well
251 was washed three times with 250 μl of sterilized physiological saline. The plates were shaken
252 vigorously to remove all planktonic bacteria. Then 200 μl of 99% methanol was added into
253 each well to fix the attached bacteria. After 15 min, the plates were emptied and dried with a
254 hair drier. Then, each well was stained with 200 μl of 2% crystal violet solution for 5 min, and
255 rinsed off the excess stain with running tap water. After drying the plates with a hair drier, 160
256 μl of 33% (v/v) glacial acetic acid was added to each well to re-dissolve the dye bound to the
257 adherent bacteria. Finally, OD was recorded with an automated microtiter plate reader at 570
258 nm. Based on the OD values of bacterial films, the results of the microtiter plate test were
259 classified into four categories, non-adherent ($OD \leq OD_C$), weakly adherent ($OD_C < OD \leq 2 \times$
260 OD_C), moderately adherent ($2 \times OD_C < OD \leq 4 \times OD_C$) and strongly adherent ($OD > 4 \times OD_C$),

261 where OD_C is the cut-off OD equal to three times of standard deviation (SD) over the mean
262 OD of the negative control.
263
264 **2.9 Determination of antibiotic adsorption to KGM**
265 The antibiotic adsorption of KGM and degraded products was determined as follows. The
266 KGM samples were dissolved at 5 g/l with Milli-Q water under constant stirring overnight.
267 Each of the antibiotics was dissolved completely at 1 mg/ml to the KGM solution with vigorous
268 agitation. The solution (7 ml) was transferred into a dialysis tubing (MWCO 3.5 kDa, Spectrum
269 Laboratories, USA) and placed into a beaker, which contained 28 ml Millie-Q water agitated
270 constantly with a magnetic stirrer at room temperature. After dialysis for 24 h, antibiotic
271 concentration in the dialyzing water was analyzed by high performance liquid chromatography
272 (HPLC). The HPLC system consisted of an Agilent 1100 series equipped with a UV-VIS
273 detector and an auto-sampling equipment (Agilent 1200 Series) and a C_{18} analytical column
274 (250 mm×4.6 mm×5 μm, Alltech, USA).
275
276 Penicillin analysis was according to a reported method (Benito-Peña, Partal-Rodera, León-
277 González, & Moreno-Bondi, 2006) with minor modifications. The standard solution was
278 prepared by dissolving 122.8 mg penicillin G standard (Sigma, St. Louis, Mo, USA) in 0.9%
279 NaCl solution in a 10 ml volumetric flask and was serial diluted with 0.9% NaCl solution. The
280 HPLC mobile phase consisted of 0.02 mol/l NaH_2PO_4 (38%) and Methanol (62%) (pH of
281 NaH_2PO_4 solution adjusted to 3.1 with phosphoric acid), flowing at 1.0 ml/min. The sample
282 injection volume was 10 μl, column temperature 25°C and UV detection at 242 nm.
283
284 Tetracycline analysis was performed based on a reported procedure (Shariati, Yamini, &
285 Esrafili, 2009) with modifications. Tetracycline standard (Sigma, St. Louis, Mo, USA) was

286 dissolved in Millie-Q water (100 mg in 10 ml) in a volumetric flask and the solution was diluted
287 in a series. The HPLC mobile phase consisted of 0.02 mol/l NaH_2PO_4 : Methanol (47% : 53%),
288 flowing at 1.2 ml/min. The sample injection volume was 10 µl, column temperature 25 °C and
289 US detection at 270 nm.

290

291 **2.10 Statistical analysis**

292 MIC and MBC assays were conducted with four replicates. *Kruskal-Wallis* test and *Nemenyi*
293 test were used for the MIC and MBC data analysis and the median was taken for the results.
294 Other experiments were performed in triplicate and the results were averaged. Student's *t* test
295 was applied for the comparison of OD values and antibiotic concentrations. The data analysis
296 was performed using SPSS 23.0 program.

297

298 **3. Results and discussion**

299 **3.1 Intrinsic viscosity and MW distribution of KGM and degraded products**

300 Table 2 presents the intrinsic viscosity and MW distribution results of KGM and partially
301 degraded KGM as well as GOS and inulin used in the experiments (GPC profiles for MW in
302 supplemental data). The intrinsic viscosity of KGM was significantly lower after the US
303 treatment, and the major MW peaks showed a general shift from high to low MW and the
304 percentage (relative peak area) of high MW components decreased. The acid-hydrolyzed KGM
305 product KGM-AH was relatively homogenous with a single low MW peak at 1369 Da.

306

307 **Table 2** The intrinsic viscosity and molecular weight of GOS, inulin, KGM and partially
308 degraded KGM (GPC profiles in Supplemental data Fig. 1).

Sample	Intrinsic viscosity (dL/g)	MW (Da)	% Area
KGM	1.2457	1.679×10^8	29.62
		7.066×10^7	33.95

KGM-US	0.5392	1.169×10^8	22.46
		1.301×10^6	77.54
KGM-AH	ND	1369	98.89
GOS	ND	530	97.91
Inulin	ND	3463	~100

309

310 **3.2 Effects of KGM and degraded products on sensitivity of bifidobaceria to antibiotics**

311 Table 3 shows the results of MIC and MBC tests of antibiotics on five bifidobacterial strains
312 cultivated in the RCM culture medium supplemented with various poly- and oligosaccharides.
313 The MIC and MBC values of a given antibiotic varied with the bacterial strains. Except for a
314 few cases, GOS and inulin (at 5 g/l) had very small effects on the MIC and MBC values of four
315 antibiotics compared with those of the control. For most bifidobacterial strains in the control,
316 penicillin was the most potent with the lowest MIC values (all ≤ 1 μg/ml) and MBC values (all
317 ≤ 16 μg/ml). The MIC value of penicillin was increased most dramatically by KGM-US at 5
318 g/l to > 512 μg/ml for all five bifidobacterial strains. On the other hand, the native KGM
319 significantly increased the MBC value of penicillin for all strains.

320

321 The MIC value of enrofloxacin varied in a much wider range than penicillin from 1 to 128
322 μg/ml with the bifidobacterial strains. In comparison, *B. bifidum* and *B. breve* were less
323 sensitive to enrofloxacin with higher MIC values (64-128 μg/ml) than other strains. KGM-US
324 at 5 g/l increased the MIC of *B. adolescentis* (64-128 μg/ml versus 1-2 μg/ml for the control)
325 and *B. bifidum* (512 μg/ml versus 64-128 μg/ml for the control).

326

327 The MIC values of tetracycline for the bifidobacterial strains varied from below 1 μg/ml to 32
328 μg/ml. *B. adolescentis* was the most sensitive to both enrofloxacin and tetracycline with the
329 lowest MIC values of 1-2 μg/ml compared with those for other bacterial strains. KGM (at 5 g/l)

330 decreased the MIC but increased the MBC significantly to ≥ 512 μg/ml for most of the

331 bifidobacterial strains.

332

333

334 All five bifidobacterial strains were relatively resistant to streptomycin with high MIC and

335 MBC values. The phenomenon is consistent with that in a previous study (Kheadr, Bernoussi,

336 Lacroix, & Fliss, 2004). Streptomycin inhibited the bifidobacteria at high concentrations from

337 16 to >512 μg/ml. *B. infantis* was most resistant to streptomycin with high MIC and MBC

338 values > 512 μg/ml. Nevertheless, KGM and KGM-US (5 g/l) increased the MIC and MBC

339 values for other four strains by 2-16 folds in most cases.

340

341 **Tables 3** Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) in

342 μg/ml of antibiotics on five strains of *Bifdobacterium* in RCM supplemented with native, and

343 degraded KGM, GOS and insulin (all at 5 g/L if not specified otherwise)

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
Control (none)	1-2	2	<1	<1	<1	32-128	256	256-512
GOS	2	2	<1	<1	1	8	128	128
Inulin	2	1-2	<1	<1	1	8	128	128
KGM	<1	>512	<1	>512	<1	>512	>512	>512
KGM-US	64-128	>512	>512	>512	1-2	>512	>512	>512
KGM-AH	16	ND	<1	ND	<1	ND	256-512	ND
KGM-US (0.5 g/l)	1	16	<1	1	<1	128	128	256
KGM-US (2 g/l)	1	64	4-8	32	<1	64	128	>512
B. bifidum								
Control (none)	64-128	32	<1	8	8	128	128	128
GOS	128-256	512	<1	1	16-32	32	8	16
Inulin	128-256	512	<1	1	32	64	32	16-32
KGM	8	>512	<1	>512	<1	>512	16	512
KGM-US	512	>512	>512	>512	2	128	256-512	>512
KGM-AH	256	ND	<1	ND	8	ND	128	ND
KGM-US (0.5 g/l)	32	32-64	1	1-4	2	128	16	32
KGM-US (2 g/l)	128	32-64	4	32	1	64	32	64
B. breve								
Control (none)	64	512	1	8	8	4-16	16	16-32
GOS	16-32	128	<1	4	1	4	16	16-32
Inulin	16	128	<1	16	1	4	32-64	32-64
KGM	<1	256	1	512	<1	512	2	64
KGM-US	64	>512	>512	>512	64-128	64-128	128-256	>512
KGM-AH	64	ND	2	ND	8	ND	16-32	ND
B. infantis								
Control (none)	4-8	32	<1	8-16	32	16	>512	>512
GOS	8	8-16	<1	<1	32	64	>512	>512

Inulin	4	8-16	<1	<1	16-32	64	>512	>512
KGM	4-8	256	<1	512	8	512	>512	>512
KGM-US	8	>512	>512	>512	8	32-64	>512	>512
KGM-AH	32	ND	<1	ND	16	ND	>512	ND
B. longum								
Control (none)	4	4-16	<1	8-16	1-2	32-128	64	32-512
GOS	8	8-16	<1	2-4	2-4	8	32	32
Inulin	8	8	<1	2-4	4	8	128	256-512
KGM	4	>512	<1	>512	<1	>512	>512	>512
KGM-US	4	>512	>512	>512	2	16	>512	>512
KGM-AH	8	ND	<1	ND	1-2	ND	128	ND

344 *Note*: Each data point is the median of four replicates. ND: Not determined.
345

346 Supplemental data Table 1 shows the relative protective effects of native and partially degraded
347 KGM by comparison of the MIC and MBC values in Table 3. KGM-US was the most effective,
348 followed by the native KGM, in protecting the bifidobacteria against the inhibition of penicillin
349 and streptomycin. KGM-US at 5 g/l increased both the MIC and MBC of penicillin for all five
350 bacterial strains by more than 64-fold, while KGM at 5 g/l increased the MBC of penicillin by
351 more than 8-fold for *B. infantis* and by more than 64-fold for other four strains. Except for *B.*
352 *infantis*, KGM or KGM-US also increased the MIC and MBC of streptomycin by 7-fold.
353 However, KGM and KGM-US showed little effect on the MIC value of enrofloxacin and
354 tetracycline but significant effect for enhancing the MBC of the two antibiotics for most
355 bifidobacterial strains.
356
357 Since KGM-US showed the most consistent and notable protective effects, it was also tested
358 at two lower concentrations, 0.5 and 2 g/l for *B. adolescentis* and *B. bifidum* (Table 1) and other
359 three bifidobacteria (Supplemental data Table 2). The MIC and MBC values usually increased
360 with the concentration increase from 0.5 to 5 g/l, indicating a dose-dependent effect.
361

362 **3.3 Effects of KGM, GOS and inulin on bifidobacterial growth**

363 As shown in Fig. 1, the effects of KGM, GOS and inulin on the bifidobacterial growth varied
364 with the bacterial strains. The prebiotic reference GOS improved the growth of four bacterial

365 strains, *B.adolescentis* (Fig. 1A), *B. breve* (Fig. 1C), *B. infantis* (Fig. 1D) and *B. longum* (Fig.
366 1E) at relative high concentrations. Inulin had a marginal effect, either positive or negative, on
367 most bacteria strains; KGM showed a slightly negative effect on most bacterial strains. The
368 acid hydrolyzed KGM (KGM-AH) only improved the growth of *B. bifidum* and *B. breve* (Fig.
369 1B-C). All the poly- and oligo-saccharides had little effect on *B. infantis* and *B. longum* (Fig.
370 1D-E). In summary, KGM and its degraded products had very small influence on the growth
371 of most bifidobacterial strains. Although inulin is widely recognized as a prebiotic
372 carbohydrate rich of fructooligosaccharides (FOS), it did not support the bifidobacterial growth.
373 Similarly, in a previous study, only eight out of the 55 *Bifidobacterium* strains could utilize
374 inulin as carbon source for growth (Rossi et al., 2005). Yang et al. (2017) also reported that
375 KGM barely supported the growth of *Lactobacilli* and *Bifidobacteria*.
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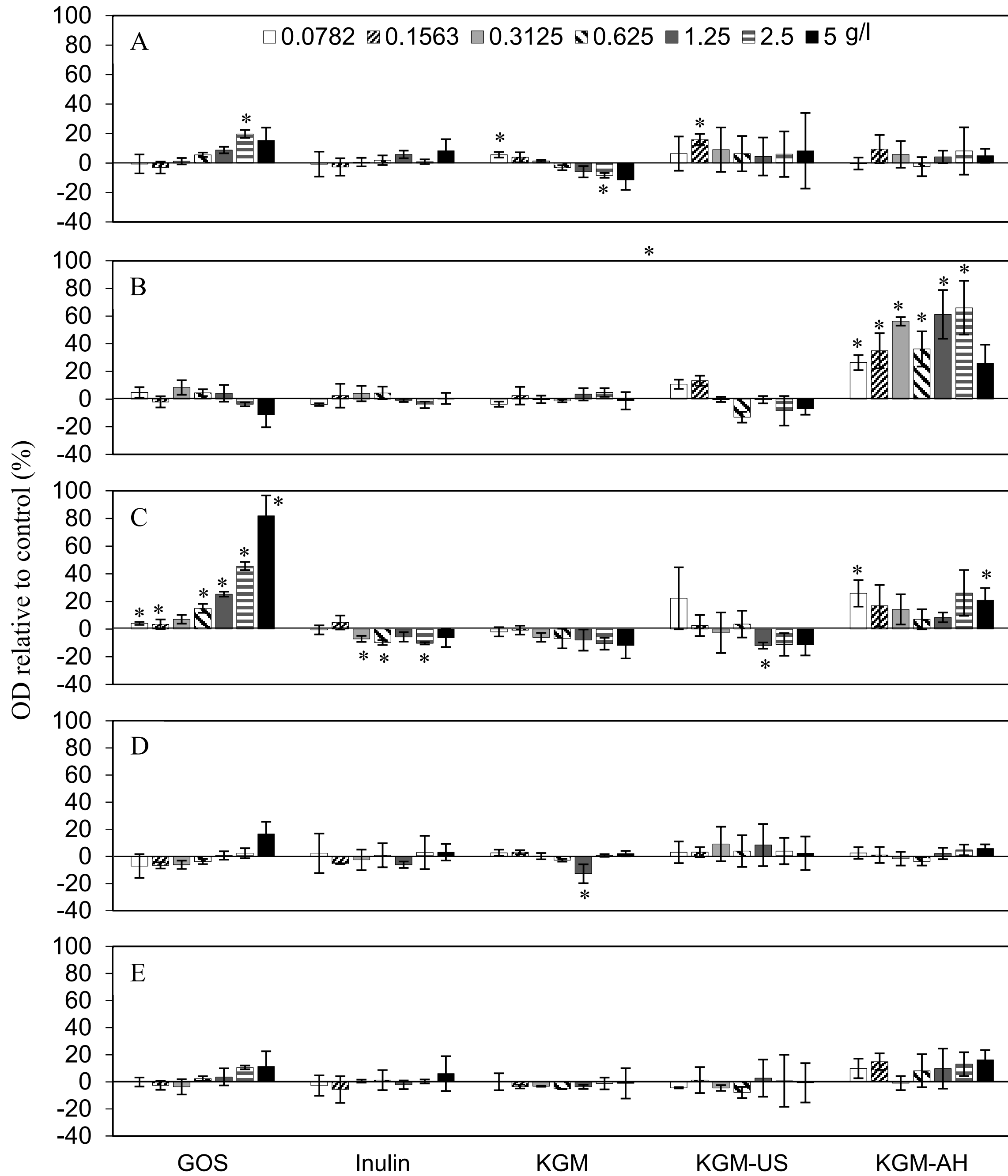

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387 **Fig. 1** Effects of GOS, inulin, native KGM and partially degraded KGM on growth of five
388 bifidobacterial strains, *B.adolescentis* (A), *B. bifidum* (B), *B. breve* (C), *B. infantis* (D) and *B.*
389 *longum* (E). (Inoculum 4×10^5 colony forming unites (cfu) in 200 μl; incubation 48 h. Error bars
390 for SD (*n* =3); *: significant difference ($p < 0.05$) compared with the control).

391

392 **3.4 Formation of biofilm in presence of KGM and KGM-US**

393 A possible mechanism for the protection of KGM or KGM-US against antibiotic inhibition is
394 the formation of a viscous layer surrounding the bacterial cell by the high MW polysaccharide,
395 which acts as a barrier to the antibiotic molecules. On the other hand, the viscous layer can also
396 block or slowdown the transfer of nutrients to the bacterial cell. As reported previously
397 (Fernandes et al., 2012), the inhibitory effect of chitooligosaccharides (COS) at a relatively
398 high concentration of 10 g/l on probiotic bacteria including *Lactobacilli* and *Bifidobacteria*
399 was attributed to the resistance to nutrient transport created by the COS surrounding or
400 covering the bacteria cell. Moreover, the viscous layer surrounding the bacterial cell can
401 enhance cell adhesion and formation of biofilms on solid surfaces. Bacterial cells in biofilm
402 are also more resistant to antibiotics (Stewart & William Costerton, 2001). KGM was chosen
403 for the biofilm test because of its high viscosity while KGM-US was chosen because of its
404 most significant protection for the bifidobacteria. As shown in Table 4, KGM and KGM-US
405 only increased the adherence of *B. infantis* to the inner surface of glass tube and had no effect
406 on other four strains. However, the tube test was not so reliable for quantifying the biofilms for
407 several reasons (Christensen et al., 1985). The microtiter-plate test showed more positive
408 results with strong or moderate adherence of bacteria to the polystyrene surface. Both KGM
409 and KGM-US increased the biofilm formation for *B. adolescentis*, *B. infantis* and *B. longum*
410 by one degree (from "++"to"+++"), though KGM caused a slight reduction of biofilm
411 formation for *B. bifidum*.

412

413 **Table 4** Adhesion ability of five strains of *Bifidobacteria* by tube and microtiterplate tests

Test	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Tube test					
Control (none)	-	+	-	-	-
KGM (5 g/l)	-	+	-	+	-
KGM-US (5 g/l)	-	+	-	+	-
Microtiter-Plate test					
Control (none)	++	+++	++	++	++
KGM (5 g/l)	+++	++	+++	+++	+++
KGM-US (5 g/l)	+++	+++	++	+++	+++

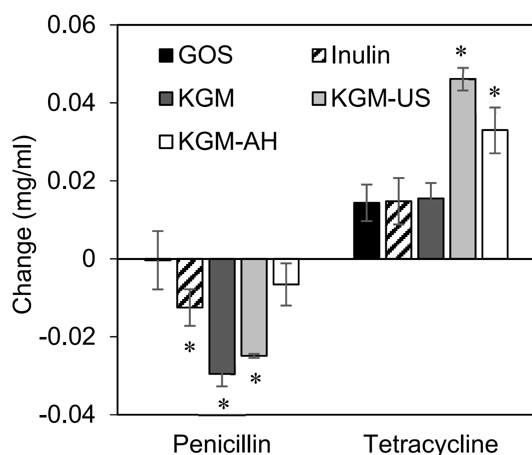
414 *Note*: -: not adherent, +: slightly adherent, ++ moderately adherent, and +++ strongly adherent,
415 compared to the negative control. Cultured for 2 d at 37 °C.
416
417

418 The result of microtiter-plate test may be more relevant to this study because the protective
419 effects were conducted in polystyrene micro-titer plates. The improved biofilm formation of
420 most bacterial strains with the addition of 5 g/l KGM and KGM-US was quite consistent with
421 the protective effect of KGM and KGM-US against antibiotics. The different results from glass
422 tube and microtiter-plate tests indicated that the material property influences the adherent
423 ability of the bacteria. To mimic the large intestine environment for microbiota, some
424 researchers have applied the intestinal epithelial cell model established by colonic carcinoma
425 (Caco-2) cells to assess the adherence of bacteria in intestine (Parkar et al., 2010).
426

427 **3.5 Adsorption of antibiotics by KGM and KGM-US**

428 Another possible mechanism for the protective effect of KGM and degraded products against
429 antibiotic inhibition of the bifidobacteria is the adsorption of the antibiotics to the
430 polysaccharides, thus decreasing the free antibiotic concentration in the culture medium (Fig.
431 2). Because penicillin was the most affected and tetracycline the least affected among the four
432 antibiotics by KGM and KGM-US based on the MIC and MBC assays, the two antibiotics were

433 chosen for this test via the dialysis experiment. Considering the water binding capacity of
434 polysaccharide, theoretically, the final concentration of antibiotics in the water outside of the
435 dialysis tubing should be at least 0.2 mg/ml if polysaccharide adsorbs no antibiotics. Penicillin
436 and tetracycline were proven to traverse the membrane freely because there was no significant
437 difference between the concentration of control group and the theoretical concentration (0.2
438 mg/ml) for penicillin or tetracycline by the *t* test ($p > 0.05$).



439

440 **Fig. 2** The change of antibiotic concentration outside the dialysis tubing (initially containing
441 5 g/l of poly- or oligo-polysaccharide and 1 mg/ml of antibiotics; dialysis against water at
442 20 °C for 24 h). (Error bars for SD, n =3; *: significant difference at $p < 0.05$ compared with
443 control).

444

445 As shown in Fig. 2, the concentrations of penicillin in KGM, KGM-US and inulin groups were
446 significantly lower than in the control, which confirmed the adsorption of penicillin by these
447 PS molecules. KGM showed the highest adsorption capacity with about 0.03 mg/ml. In contrast,
448 the concentrations of tetracycline in all test groups were higher than in the control, especially
449 significant in the KGM and degraded KGM groups. The higher concentration of tetracycline
450 observed may be attributed to the water absorbability of KGM and degraded products.
451 Moreover, the sharply different absorption ability of KGM for penicillin and tetracycline may
452 be attributed to the different molecular properties of the two antibiotics, especially the polarity

453 as penicillin is very polar and soluble in water while tetracycline is less polar and less soluble
454 in water (Chlou, Malcolm, Brinton, & Klle, 1986; Soren, 2003).
455
456 The results of adsorption experiments are in general agreement with the finding from the above
457 that KGM and KGM-US had a significant protective effect on the bifidobacteria against
458 penicillin but little effect against tetracycline. However, a quantitative correlation could not be
459 found between the adsorption concentrations and the changes in MIC or MBC due probably to
460 the simplistic adsorption experimental system and the tedious MIC and MBC assay procedure.
461 Therefore, the protective effect of KGM and KGM-US against some of the antibiotics can be
462 partially attributed to the adsorption of these antibiotics to the polysaccharides thereby
463 decreasing the concentration of free antibiotic in the system.
464

465 **4. Conclusions**

466 The present study has revealed the protective effect of KGM, especially the US-degraded KGM
467 on bifidobacteria against some common antibiotics including penicillin and streptomycin.
468 Partially degraded KGM by high intensity ultrasound was more effective than the native KGM
469 and the low molecular weight, acid-hydrolyzed KGM. Two prebiotic standards GOS and inulin
470 showed no significant protective effect. The protective effect of KGM or KGM-US on the
471 bifidobacteria may be attributed to the adsorption of antibiotics and the formation of a viscous
472 layer surrounding the bacteria by the polysaccharides. On the other hand, the weak or no
473 protection by the acid hydrolyzed KGM, inulin and GOS with much lower MW was probably
474 because they could not form the viscous layer surrounding the bacteria. However, the present
475 study has only detected the protective effect of KGM in the pure cultures of a few bifidobacteria.
476 As the protective effect varied with the bacterial strains, further investigation should be carried

477 out in mixed cultures of gut microflora to evaluate the potential application in human gut
478 microbiota.
479

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486

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631

632 **Supplemental data**

633

634 **Table 1** The protective effects of native, and degraded KGM, GOS and insulin (all at 5 g/L if
635 not specified otherwise) bifidobacteria against antibiotic inhibition based on results of MIC
636 and MBC assays presented in Table 3.

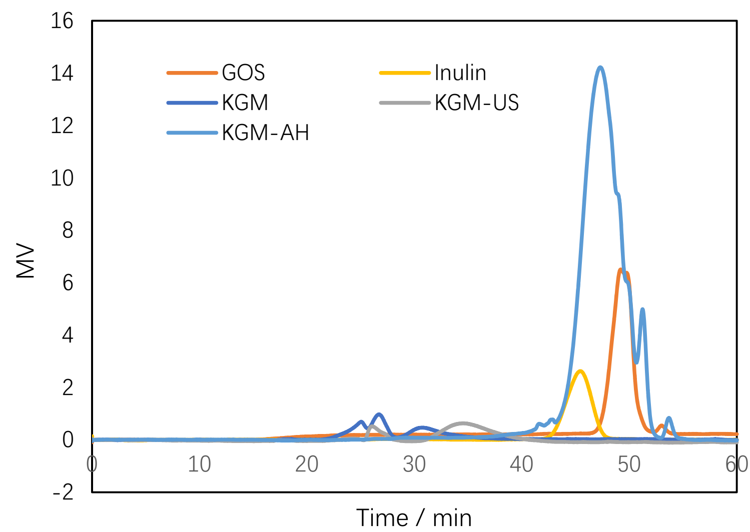
OS/PS (5 g/l or	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
specified)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
KGM	-	+++	-	+++	-	++	+	+
KGM-US	++	+++	+++	+++	+	++	+	+
KGM-AH	++	ND	-	ND	-	ND	+	ND
KGM-US (0.5 g/l)	-	+	-	+	-	-	-	-
KGM-US (2 g/l)	-	++	+	++	-	-	-	+
B. bifidum								
KGM	-	++	-	+++	-	+	-	+
KGM-US	+	++	+++	+++	-	-	+	+
KGM-AH	+	ND	-	ND	-	ND	+	ND
KGM-US (0.5 g/l)	-	+	+	-	-	-	-	-
KGM-US (2 g/l)	-	+	+	+	-	-	-	-
B. breve								
KGM	-	+	-	+++	-	+++	-	+
KGM-US	-	+	+++	+++	++	++	++	++
KGM-AH	-	ND	+	ND	-	ND	+	ND
B. infantis								
KGM	-	++	-	++	-	++	-	-
KGM-US	-	++	+++	+++	-	+	-	-
KGM-AH	+	ND	-	ND	-	ND	-	ND
B. longum								
KGM	-	+++	-	+++	-	++	++	+
KGM-US	-	+++	+++	+++	-	-	++	+
KGM-AH	+	ND	-	ND	-	ND	+	ND

637 *Note:* -: no positive effect; +: value ≤ 8 times of control; ++: 8 < value ≤ 64 times of control;
638 +++: value > 64 times of control; ND: not determined.

Tables 2 Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) in µg/ml of antibiotics on five strains of *Bifdobacterium* in RCM supplemented with native, and degraded KGM, GOS and insulin (all at 5 g/L if not specified otherwise)

OS/PS (5 g/l or	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
specified)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
Control (none)	1-2	2	<1	<1	<1	32-128	256	256-512
GOS	2	2	<1	<1	1	8	128	128
Inulin	2	1-2	<1	<1	1	8	128	128
KGM	<1	>512	<1	>512	<1	>512	>512	>512
KGM-US	64-128	>512	>512	>512	1-2	>512	>512	>512
KGM-AH	16	ND	<1	ND	<1	ND	256-512	ND
KGM-US (0.5 g/l)	1	16	<1	1	<1	128	128	256
KGM-US (2 g/l)	1	64	4-8	32	<1	64	128	>512
B. bifidum								
Control (none)	64-128	32	<1	8	8	128	128	128
GOS	128-256	512	<1	1	16-32	32	8	16
Inulin	128-256	512	<1	1	32	64	32	16-32
KGM	8	>512	<1	>512	<1	>512	16	512
KGM-US	512	>512	>512	>512	2	128	256-512	>512
KGM-AH	256	ND	<1	ND	8	ND	128	ND
KGM-US (0.5 g/l)	32	32-64	1	1-4	2	128	16	32
KGM-US (2 g/l)	128	32-64	4	32	1	64	32	64
B. breve								
Control (none)	64	512	1	8	8	4-16	16	16-32
GOS	16-32	128	<1	4	1	4	16	16-32
Inulin	16	128	<1	16	1	4	32-64	32-64
KGM	<1	256	1	512	<1	512	2	64
KGM-US	64	>512	>512	>512	64-128	64-128	128-256	>512
KGM-AH	64	ND	2	ND	8	ND	16-32	ND
KGM-US (0.5 g/l)	8	32	≤ 1	8	<1	8	8	16
KGM-US (2 g/l)	2-8	32-64	1-2	64	2	16	16	32
B. infantis								
Control (none)	4-8	32	<1	8-16	32	16	>512	>512
GOS	8	8-16	<1	<1	32	64	>512	>512
Inulin	4	8-16	<1	<1	16-32	64	>512	>512
KGM	4-8	256	<1	512	8	512	>512	>512
KGM-US	8	>512	>512	>512	8	32-64	>512	>512
KGM-AH	32	ND	<1	ND	16	ND	>512	ND
KGM-US (0.5 g/l)	8-32	64	<1	2-4	8	32	>512	>512
KGM-US (2 g/l)	8-32	64	4-16	32	8	32	>512	>512
B. longum								
Control (none)	4	4-16	<1	8-16	1-2	32-128	64	32-512
GOS	8	8-16	<1	2-4	2-4	8	32	32
Inulin	8	8	<1	2-4	4	8	128	256-512
KGM	4	>512	<1	>512	<1	>512	>512	>512
KGM-US	4	>512	>512	>512	2	16	>512	>512
KGM-AH	8	ND	<1	ND	1-2	ND	128	ND
KGM-US (0.5 g/l)	8	64-128	<1	8	1-2	64	512	>512
KGM-US (2 g/l)	4	32-64	4	16-32	1-2	64	512	>512

Note: Each data point is the median of four replicates. ND: Not determined.



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644 **Fig. 1** GPC profiles (molecular weight distributions) of KGM and degraded products.