

# Antioxidative capacity produced by *Bifidobacterium*- and *Lactobacillus acidophilus*-mediated fermentations of konjac glucomannan and glucomannan oligosaccharides

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## Abstract

**BACKGROUND:** Konjac glucomannan (KGM) has been shown to stimulate the growth of bifidobacteria and lactobacilli in the human and rat colon. This study investigated the antioxidative effects produced after 48 h *in vitro* fermentation of unhydrolysed KGM and two hydrolysed KGM fractions (KH1 and KH2 with degree of polymerisation 10 and 5 respectively) by *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. longum* and *Lactobacillus acidophilus* respectively. The inhibitory effect on conjugated diene formation, ferric-chelating capacity,  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical-scavenging ability and thiobarbituric acid-reactive substances (TBARS) concentration produced by these fermentations were compared with those of oligofructose (OF) fermentation.

**RESULTS:** The fermentation of KGM by each bacterial strain produced higher ferric-chelating capacity of the culture supernatant compared with KH2 or OF fermentation. In contrast, the fermentation of KGM by each bacterial strain led to lower inhibition of conjugated diene formation and lower radical-scavenging ability compared with KH2 fermentation. The fermentation of KH2 produced the lowest amount of TBARS.

**CONCLUSION:** The fermentation of unhydrolysed KGM by colonic lactic acid bacteria *in vitro* produced antioxidative capacity mainly by preventing the initiation of ferrous ion-induced peroxidation, whereas the fermentation of konjac oligosaccharides did so by increasing the radical-scavenging ability and eliminating lipid peroxide formation.

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**Keywords:** antioxidative; konjac glucomannan; *Bifidobacterium*; *Lactobacillus acidophilus*; fermentation; oligofructose

## INTRODUCTION

Epidemiological studies have shown that a diet rich in fibre is beneficial for preventing diseases associated with free radical damage, such as heart disease<sup>1</sup> and cancer.<sup>2</sup> One of the mechanisms by which undigestible carbohydrates prevent carcinoma is to stimulate the growth of colonic lactic acid bacteria, which have been shown to exert anticarcinogenic<sup>3–6</sup> and antioxidative<sup>7–9</sup> effects. However, the antioxidative effect of dietary fibre or its metabolites is rarely examined. There is only a report indicating that fermentation of oligofructose by human faeces can reduce the genotoxicity of faecal water to colon cells,<sup>10</sup> which suggests that fermentation of oligofructose produces antioxidative ability.

Konjac glucomannan (KGM), a viscous, water-soluble dietary fibre obtained from konjac plant root powder, is commonly used as a gelling agent in Asia. KGM is a beneficial dietary adjunct for treatment of metabolic syndromes related to free radical damage, such as hypercholesterolaemia, glucose intolerance and diabetes.<sup>11,12</sup> In addition, KGM was recently found to stimulate the growth of bifidobacteria and lactobacilli in both animals<sup>13</sup> and healthy adults.<sup>14</sup> Therefore KGM could reduce oxidative stress through fermentation by bifidobacteria and lactobacilli.

Although KGM exerts many beneficial physiological functions, its high viscosity limits its application in foods. Downsizing of the glucomannan polymer to smaller molecules decreases its viscosity and may expand its application. In a previous study we

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(Received 5 September 2007; revised version received 25 December 2007; accepted 6 January 2008)

Published online 31 March 2008; DOI: 10.1002/jsfa.3226

demonstrated that a KGM hydrolysate (degree of polymerisation (DP) 12) exerted stronger prebiotic effects than its unhydrolysed parent polymer in Balb/c mice.<sup>13</sup> However, antioxidative effects produced during fermentation of konjac glucomannan of various DP by common intestinal lactic acid bacteria remain to be investigated. Therefore konjac hydrolysate was prepared and fractionated into two oligosaccharide fractions denoted KH1 and KH2, in which the DP of glucomannan was 10 and 5 respectively. Indices of antioxidative ability were measured after 48 h lactic acid bacteria-mediated fermentation of KGM, KH1, KH2 and oligofructose (OF).

## MATERIALS AND METHODS

### Test carbohydrates

The KGM powder (Fukar Trading Co., Taipei, Taiwan) contained 34 g protein, 38 g fat, 29 g ash and 86 g moisture  $\text{kg}^{-1}$  as analysed by AOAC<sup>15</sup> methods. The OF was a commercial syrup consisting of 550 g total carbohydrates and 450 g water  $\text{kg}^{-1}$  as indicated by the manufacturer (Taiwan Sugar Co., Taipei, Taiwan).

### Preparation and fractionation of glucomannan hydrolysate

Konjac oligosaccharides were prepared from the raw KGM hydrolysate by the following method. The konjac powder ( $25 \text{ g L}^{-1}$ ) dissolved in HCl ( $0.2 \text{ mol L}^{-1}$ ) in a round-bottomed flask was boiled in a reflux condenser for 40 min and subsequently condensed *in vacuo* to one-tenth of its original volume. Ethanol was then added to this raw KGM hydrolysate to give a  $750 \text{ g kg}^{-1}$  ethanolic solution, from which a pellet denoted KH1 was obtained after centrifugation at  $10\,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The remaining supernatant was made up to a  $900 \text{ g kg}^{-1}$  ethanolic solution, which was then centrifuged at  $17\,500 \times g$  for 30 min at  $4^\circ\text{C}$  to yield a pellet denoted KH2. KH1 and KH2 were repeatedly rinsed with ethanol to remove residual acid, lyophilised and stored in desiccators until use.

### Characterisation of KH1 and KH2

#### Determination of degree of polymerisation

Total and reducing sugar contents were analysed by the method of Dubois *et al.*<sup>16</sup> and the Somogyi–Nelson method<sup>17</sup> respectively. The average DP was calculated as the ratio of total sugar content ( $\text{g kg}^{-1}$ ) to reducing sugar content ( $\text{g kg}^{-1}$ ).<sup>13</sup>

#### Analysis of saccharide compositions

Mono- and oligosaccharide compositions were examined by high-performance anion exchange chromatography (HPAEC), mainly following the methods of Lai *et al.*<sup>18</sup> and Vincken *et al.*<sup>19</sup> respectively. A Dionex DX 500 LC system with an ED40 detector (Dionex Co., Sunnyvale, CA, USA) was employed, with the pulse potentials of the detector set at  $E_1 = 0.05 \text{ V}$ ,  $E_2 =$

$0.75 \text{ V}$  and  $E_3 = -0.15 \text{ V}$  for durations  $t_1 = 0.4 \text{ s}$ ,  $t_2 = 0.2 \text{ s}$  and  $t_3 = 0.5 \text{ s}$  respectively.

For monosaccharide composition, 10 mg of KH1 or KH2 in a screw-capped glass tube was hydrolysed with 10 mL of  $2 \text{ mol L}^{-1}$  trifluoroacetic acid (TFA) in a boiling water bath for 4 h. The obtained hydrolysate was immediately cooled in iced water, followed by removing water and TFA *in vacuo* (Savant Speed-Vac model 100 evaporator, Savant Instruments, Inc., New York, NY, USA), redissolving in 10 mL of deionised water, removing ions with (Sigma, St Louis, MO, USA) Amberlite IRA 400 resin ( $\text{Cl}^-$  form) and then filtering through a  $0.22 \mu\text{m}$  membrane prior to HPAEC analysis. A (Dionex Co., Sunnyvale, USA) CarboPac™ PA I column ( $250 \text{ mm} \times 4 \text{ mm i.d.}$ ) with a guard column ( $50 \text{ mm} \times 4 \text{ mm i.d.}$ ) was used. The eluent was a 5:95 (v/v) mixture of  $0.20 \text{ mol L}^{-1}$  NaOH and deionised water. The flow rate was  $0.75 \text{ mL min}^{-1}$  and the sample size was  $25 \mu\text{L}$ . Data were means of triplicate measurements, calibrated using standard curves for glucose and mannose (Sigma, St Louis, MO, USA).

For oligosaccharide composition, 10 mg of KH1 or KH2 was dissolved in 10 mL of deionised water in a screw-capped glass tube, followed by removing ions with (Sigma, St Louis, MO, USA) Amberlite IRA 400 resin ( $\text{Cl}^-$  form) and filtering through a  $0.22 \mu\text{m}$  membrane prior to HPAEC analysis. A (Dionex Co., Sunnyvale, USA) CarboPac™ PA 100 column ( $250 \text{ mm} \times 4 \text{ mm i.d.}$ ) with a guard column ( $50 \text{ mm} \times 4 \text{ mm i.d.}$ ) was used. The eluent was a mixture of  $0.2 \text{ mol L}^{-1}$  NaOH (A) and  $0.5 \text{ mol L}^{-1}$  sodium acetate in  $0.2 \text{ mol L}^{-1}$  NaOH (B), using a linear gradient of 100% A at 0–10 min, 50% A at 40 min and 100% A at 60 min. The flow rate and sampling size were the same as for monosaccharide analysis. Data were means of triplicate measurements, calibrated using a standard curve for maltose (Sigma) owing to its similar chromatographic properties to konjac di- and trisaccharide fragments.

### Bacteria

We compared the stimulatory effects of low concentrations ( $5 \text{ g L}^{-1}$ ) of undigestible carbohydrates on the growth of four species of bifidobacteria and *Lactobacillus acidophilus* commonly found in the human colon.<sup>20</sup> The starter *Bifidobacterium adolescentis* (ATCC 15703, BCRC 14606), *B. bifidum* (BCRC 11844), *B. breve* (ATCC 15700, BCRC 11846), *B. longum* (ATCC 15707, BCRC 11847) and *L. acidophilus* (ATCC 4356, BCRC 10695) were obtained from the Biore-source Collection and Research Centre (BCRC) of the Food Industry Research and Development Institute, Hsinchu, Taiwan. They were activated twice prior to the fermentation experiment in sterilised Lactobacilli MRS broth (Becton Dickinson and Co., Sparks, MD, USA) containing  $0.5 \text{ g L}^{-1}$  cysteine (Sigma) at  $37^\circ\text{C}$  for 48 h in an anaerobic system (BBL GasPak, Becton Dickinson and Co.; Anaerocult®, Merck, Darmstadt, Germany). The concentration of each starter organism

was then adjusted to  $10^9$  colony-forming units (CFU)  $L^{-1}$  for the fermentation experiment.

### ***In vitro* fermentation**

The basal broth comprised 5 g peptone, 5 g beef extract, 2.5 g yeast extract, 10 g dextrose, 0.5 g polysorbate, 1 g ammonium citrate, 2.5 g sodium acetate, 0.05 g magnesium sulfate, 0.025 g manganese sulfate, 1 g dipotassium phosphate and 0.5 g cysteine  $L^{-1}$ , to which the test undigestible carbohydrates were supplemented at  $0.5 g L^{-1}$ . Tubes each containing 8 mL of sterilised broth and 0.1 mL of activated starter organism ( $10^9$  CFU  $L^{-1}$ ) were incubated at  $37^\circ C$  in five anaerobic systems (BBL GasPak, Becton Dickinson and Co.; Anaerocult<sup>®</sup>, Merck). The duration of fermentation was 0, 6, 12, 24 and 48 h respectively. Three batches of fermentation were conducted for each bacterial strain.

The absorbance of the cultures was measured at 510 nm in triplicate tubes at each time point to monitor the growth of bacteria. The 48 h cultures were centrifuged at  $10\,000 \times g$  for 10 min at  $4^\circ C$  to obtain the cell-free culture supernatants. The culture supernatants were immediately lyophilised and then stored in the dark at  $-20^\circ C$  until analysis for antioxidative capacity.

### **Antioxidative properties of culture supernatants after 48 h of fermentation**

The manner in which the test undigestible carbohydrates modulated the antioxidative ability of the culture supernatants was assessed in terms of  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical-scavenging ability, chelation of ferric ion, inhibition of conjugated dienes and formation of thiobarbituric acid-reactive substances (TBARS).

#### *DPPH radical-scavenging ability*

DPPH radical-scavenging ability, an indicator of hydrogen-donating ability, was measured by the method of Shimada *et al.*<sup>21</sup> with slight modification. A 0.5 mL aliquot of culture supernatant or  $\alpha$ -tocopherol ( $10\text{ mmol } L^{-1}$ , as a positive control) and 0.125 mL of DPPH ( $1\text{ mmol } L^{-1}$  in methanol) were incubated at  $37^\circ C$  for 30 min. DPPH has a main absorbance at 517 nm, which diminishes as the hydrogen-donating capacity of the antioxidant increases. Therefore the DPPH radical-scavenging ability was calculated as follows:

$$\begin{aligned} &\text{DPPH radical-scavenging ability (\%)} \\ &= [1 - (A_{517} \text{ of sample} / A_{517} \text{ of control})] \times 100 \end{aligned}$$

#### *Ferric-chelating capacity*

Ferric-chelating capacity, an indicator of reducing peroxidation reaction induced by metal ion, was measured by the method of Dinis *et al.*<sup>22</sup> A 0.15 mL aliquot of culture supernatant was mixed with 0.555 mL of

methanol and 15  $\mu L$  of  $2\text{ mmol } L^{-1}$   $FeCl_2$ . The mixture was incubated at  $37^\circ C$  for 10 min, after which its absorbance at 562 nm was measured. The ferric-chelating capacity was calculated as follows:

$$\begin{aligned} &\text{ferric-chelating capacity (\%)} \\ &= [1 - (A_{562} \text{ of sample} / A_{562} \text{ of control})] \times 100 \end{aligned}$$

#### *Inhibition of lipid peroxidation (conjugated diene formation)*

Based on the method used by Lingnert *et al.*,<sup>23</sup> 0.5 mL of  $10\text{ mmol } L^{-1}$  linoleic acid emulsion (pH 6.6) mixed with 25  $\mu L$  of culture supernatant or  $10\text{ }\mu\text{mol } L^{-1}$   $\alpha$ -tocopherol was incubated in the dark at  $37^\circ C$  for 15 h. A 50  $\mu L$  aliquot of the reaction solution was then mixed with 1.75 mL of  $800\text{ g } L^{-1}$  methanol. The absorbance at 234 nm was measured. The inhibition of conjugated diene formation was calculated as follows:

$$\begin{aligned} &\text{inhibition of conjugated diene formation (\%)} \\ &= [1 - (A_{234} \text{ of sample} / A_{234} \text{ of control})] \times 100 \end{aligned}$$

#### *TBARS concentration*

Based on the method described by Draper and Hadley,<sup>24</sup> 0.3 mL of culture supernatant or malonaldehyde dimethyl acetal (as a standard) and 5  $\mu L$  of  $10\text{ g } L^{-1}$  butylated hydroxytoluene were mixed with 0.5 mL of  $0.02\text{ mol } L^{-1}$  thiobarbituric acid (TBA). The mixture was boiled for 30 min in the dark, then cooled to ambient temperature, after which the absorbance at 532 nm was measured. The concentration of TBA-reactive substances (TBARS) in samples was calculated with reference to the standard.

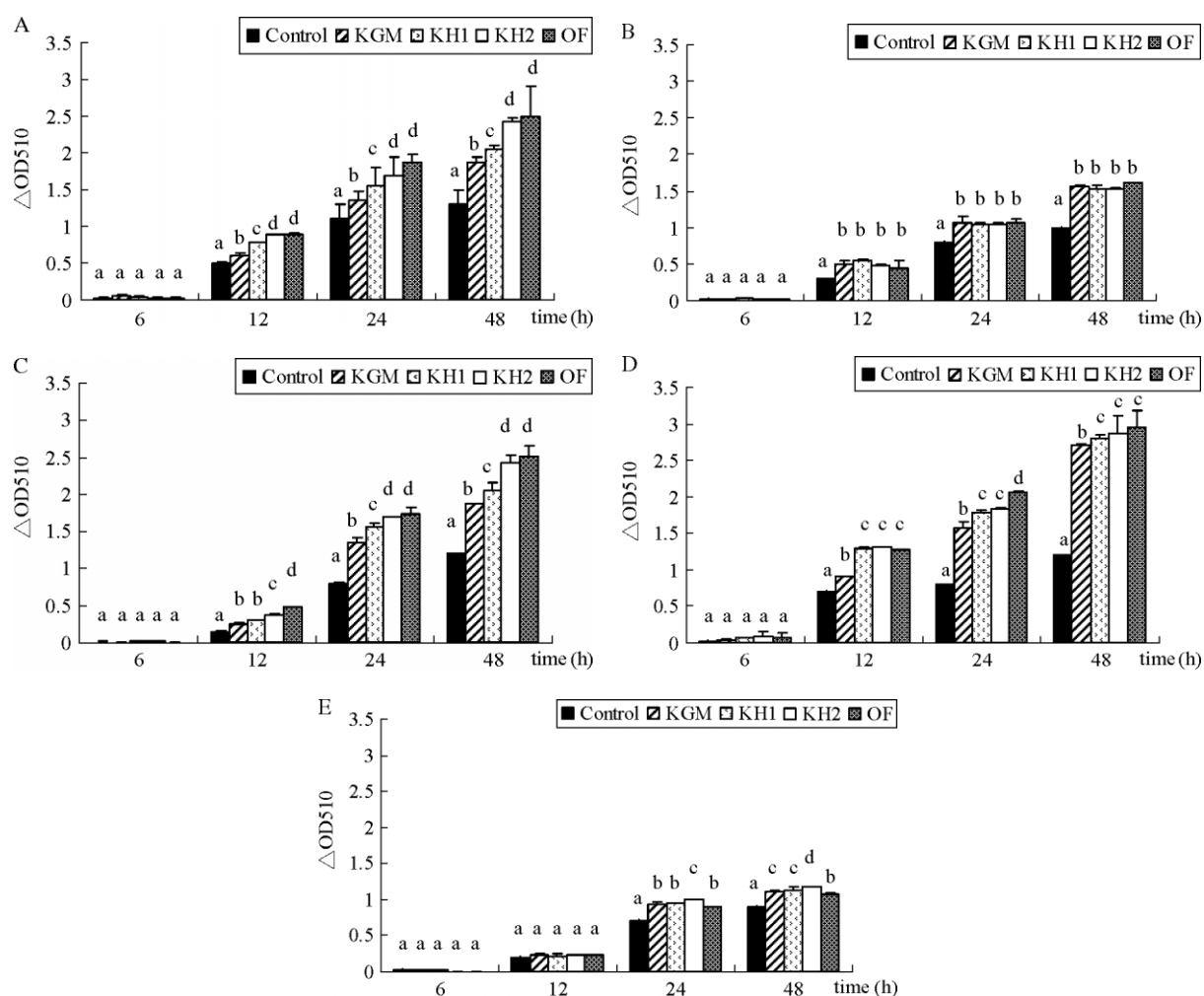
### **Statistical analysis**

Data are presented as mean  $\pm$  standard deviation and were analysed with the Statistical Package for Social Sciences (SPSS for Windows, Version 10.0, SPSS Inc., Chicago, IL, USA). Comparisons between test undigestible carbohydrates within individual bacterial cultures were made using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Different letters above bars in Figs 1 and 2 denote statistically significant differences.

## **RESULTS AND DISCUSSION**

### **Characterisation of KH1 and KH2**

The sugar compositions of KH1 and KH2 are shown in Table 1. The ratios of mannose to glucose in KH1 and KH2 were  $\sim 1.6$  and  $\sim 1.3$  respectively, similar to the data reported for unhydrolysed KGM.<sup>25</sup> The compositions of KH1 and KH2 were quite similar:  $\sim 30\text{ g}$  mannose,  $\sim 20\text{ g}$  glucose,  $\sim 560\text{ g}$  di- and trisaccharides and  $\sim 390\text{ g}$  other undetectable saccharides  $kg^{-1}$  total saccharides. Since the DP values derived by chemical analysis of KH1 and KH2 were 10 and 5 respectively, we suggest that KH2



**Figure 1.** Increases in  $OD_{510}$  of cultures in which glucomannan (KGM), konjac hydrolysate fraction 1 (KH1), konjac hydrolysate fraction 2 (KH2) and oligofructose (OF) were individually supplemented to media containing (A) *Bifidobacterium adolescentis*, (B) *B. bifidum*, (C) *B. breve*, (D) *B. longum* and (E) *Lactobacillus acidophilus* respectively. Comparisons between test undigestible carbohydrates at the same time point were made using one-way ANOVA followed by Duncan's multiple range test. Different letters above bars denote statistically significant differences between undigestible carbohydrates at the same time point ( $P < 0.05$ ).

**Table 1.** Sugar compositions of konjac hydrolysate fractions<sup>a</sup>

Sample	Sugar composition ( $g\ kg^{-1}$ total saccharides)				
	Glucose	Mannose	Di- and trisaccharides <sup>b</sup>	Others <sup>c</sup>	DP <sup>d</sup>
KH1	16 ± 0	25 ± 4	573 ± 35	386	10
KH2	24 ± 3	31 ± 4	552 ± 18	393	5

<sup>a</sup> Data are mean of three replicate determinations ± standard deviation.

<sup>b</sup> Maltose was used as a standard because of its similar chromatographic properties to the konjac di- and trisaccharides examined.

<sup>c</sup> Calculated by subtracting the detectable amount of saccharides from the total saccharides; mainly composed of low-molecular-weight polysaccharides undetectable in this study.

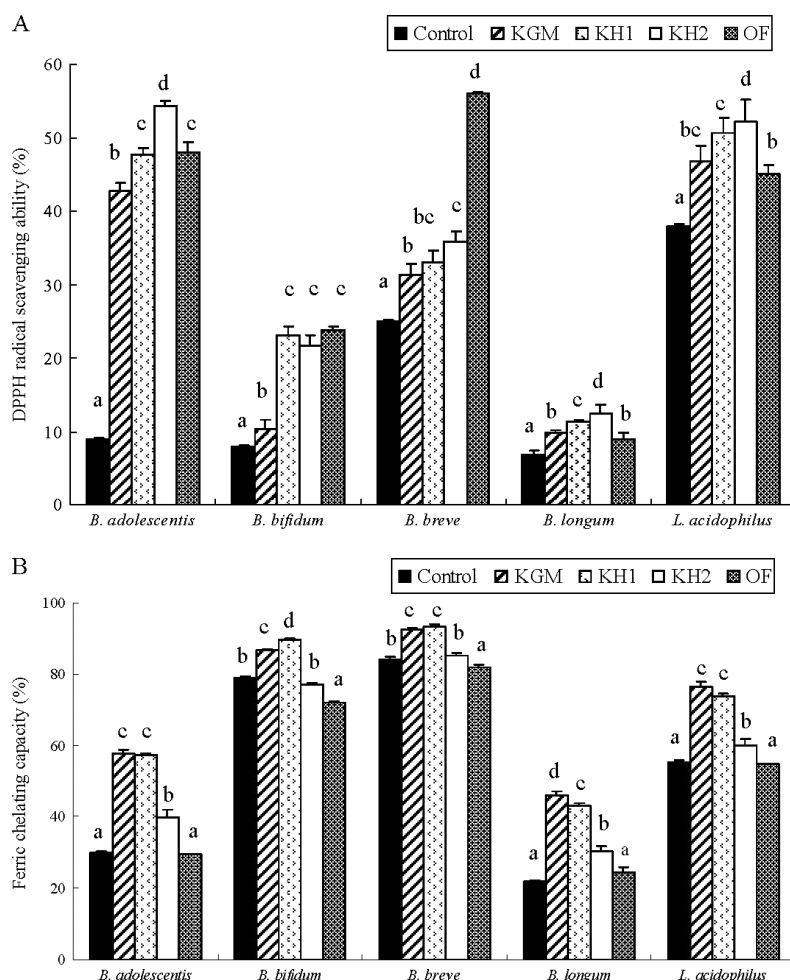
<sup>d</sup> DP (degree of polymerisation) = total sugar content ( $g\ kg^{-1}$ )/reducing sugar content ( $g\ kg^{-1}$ ).

contained greater amounts of low-molecular-weight polysaccharides than KH1.

### Bacterial growth curve

$\Delta OD_{510}$  (change in optical density at 510 nm) of each culture increased with fermentation time (Fig. 1).

Supplementation of test undigestible carbohydrates facilitated the growth of each bacterial culture, in agreement with previous studies reporting that KGM, raw glucomannan hydrolysate (DP 12 ± 1) and OF were prebiotics.<sup>13,26</sup>  $\Delta OD_{510}$  values at 6 h were similar among test carbohydrates for each species examined. The growth of *B. adolescentis* and *B. breve* increased in the order KGM < KH1 < KH2 at 24 and 48 h. Similarly, the growth of *B. longum* showed the trend KGM < KH1 ≤ KH2 at 24 and 48 h. Therefore this study agreed with our previous finding that partially hydrolysed glucomannan polysaccharide could stimulate greater *Bifidobacterium* and *L. acidophilus* growth than its parent unhydrolysed KGM.<sup>13</sup> The present study further demonstrated that KH2 (DP 5) exerted a greater prebiotic effect than KH1 (DP 10). In addition, we found that KH2 facilitated the growth of *B. adolescentis*, *B. breve*, *B. bifidum* and *B. longum* to the same extent as OF, while KGM, KH1 and KH2 were better substrates than OF for *L. acidophilus*.



**Figure 2.** Indices of antioxidative ability of culture supernatants produced after 48 h fermentation of glucomannan (KGM), konjac hydrolysate fraction 1 (KH1), konjac hydrolysate fraction 2 (KH2) and oligofructose (OF) respectively: (A) DPPH radical scavenging ability; (B) ferric chelating capacity; (C) inhibition of conjugated diene formation; (D) TBARS concentration. Comparisons between test undigestible carbohydrates with the same bacterial strain were made using one-way ANOVA followed by Duncan's multiple range test. Different letters above bars denote statistically significant differences between undigestible carbohydrates at the same time point ( $P < 0.05$ ).

### Antioxidative capacity of culture supernatant

The DPPH radical-scavenging ability of  $10 \text{ mmol L}^{-1}$   $\alpha$ -tocopherol was  $93 \pm 0.1\%$ . The DPPH radical-scavenging ability of the control group of *B. adolescentis*, *B. bifidum* and *B. longum* was equivalent to  $\sim 1 \text{ mmol L}^{-1}$   $\alpha$ -tocopherol, while that of the *L. acidophilus* culture was equivalent to  $\sim 4.3 \text{ mmol L}^{-1}$   $\alpha$ -tocopherol (Fig. 2A). Supplementation of undigestible carbohydrates generally increased the DPPH radical-scavenging ability compared with the control. In comparison with unhydrolysed KGM, both KH1 and KH2 caused greater DPPH radical-scavenging ability in all bacterial culture supernatants. In addition, KH1 and KH2 caused greater DPPH radical-scavenging ability than OF in the *B. adolescentis* and *B. longum* culture supernatants. Previous studies have shown that intracellular extracts and intact cells of *B. longum* and *L. acidophilus* produce DPPH radical-scavenging ability.<sup>27</sup> Therefore it appears that the antioxidant contributing to the DPPH radical-scavenging ability of the culture supernatant was partially derived from the bacteria. However, since

the DPPH radical-scavenging ability was not correlated with the bacterial concentration in the culture, we suggest that undigestible fibre and its metabolites were likely to modulate this antioxidative ability of the culture supernatant.

The ferric-chelating ability of the culture supernatant in the control group was in the ascending order *B. longum* < *B. adolescentis* < *L. acidophilus* < *B. bifidum* < *B. breve* (Fig. 2B). When comparisons were made between the test undigestible carbohydrates within individual bacterial strains, the ferric-chelating capacity of culture supernatants was in the order  $\text{KGM} \geq \text{KH1} > \text{KH2} > \text{OF}$ . These results imply that glucomannan of larger DP had greater ability to hinder the initiation of lipid peroxidation than glucomannan of smaller DP. In addition, OF exerted almost no enhancing effect on this antioxidative indicator as compared with the control. Therefore the physical characteristics and composition of the test carbohydrates may affect the ferric-chelating capacity of the culture supernatant.

The inhibitory effect of the culture supernatant in the control group on conjugated diene formation

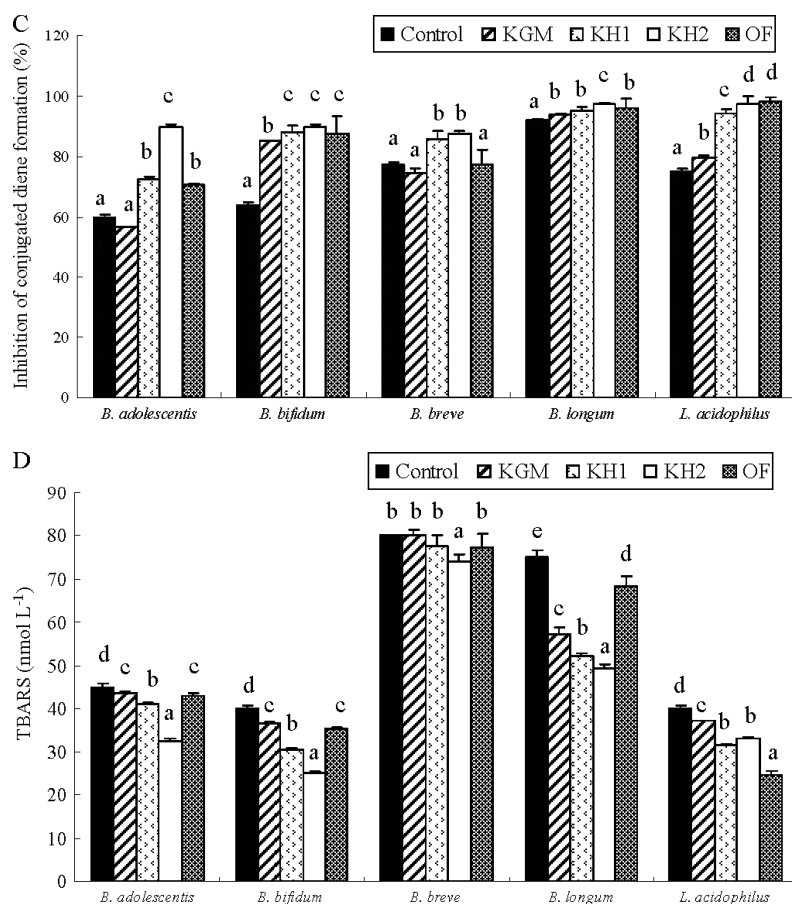


Figure 2. Continued.

was in the ascending order *B. adolescentis* < *B. bifidum* < *L. acidophilus* < *B. breve* < *B. longum* (Fig. 2C), while that of *B. longum* exceeded the effect of 10 mmol L<sup>-1</sup>  $\alpha$ -tocopherol, 92  $\pm$  0.3%. Supplementation of test undigestible carbohydrates generally decreased conjugated diene formation as compared with the control. When compared among the test undigestible carbohydrates, the inhibitory effect of culture supernatants on conjugated diene formation increased in the order KGM < KH1 < KH2 for every bacterial strain. In addition, this effect of KH2 was even greater than that of OF on *B. adolescentis* and *B. breve*.

The TBARS concentration in the culture supernatant persistently increased in the order KH2 < KH1 < KGM < control group within individual bacterial strains (Fig. 2D). These results imply that glucomannan of smaller DP promoted a greater inhibitory effect on lipid peroxidation than glucomannan of larger DP. Also, KH2 was more effective than OF in inhibiting TBARS formation in the *Bifidobacterium* cultures examined in this study.

Intact cells<sup>27</sup> and cell-free intracellular extracts<sup>7</sup> of lactic acid bacteria have been found to exert antioxidative effects. In addition, a culture supernatant of *L. acidophilus*<sup>28</sup> and extracts of bacterial cell walls<sup>29</sup> have been shown to inhibit lipid oxidation. The present study further demonstrated that additional fermentation of KGM, glucomannan oligosaccharides and OF could enhance the antioxidative ability of culture

supernatants of lactic acid bacteria through various mechanisms. Fermentation of KGM mainly produced antioxidative ability through preventing the initiation of ferrous ion-induced peroxidation in the culture supernatant, whereas KH1 fermentation reduced ferrous ion-induced peroxidation and formation of lipid peroxide products and increased radical-scavenging ability. Fermentation of KH2 and OF produced antioxidative effects similarly by increasing radical-scavenging ability and eliminating lipid peroxide formation. Furthermore, fermentation of KH2 produced greater inhibitory effects on TBARS formation than OF fermentation, probably because KH2 metabolites had greater ferric-chelating capability.

## CONCLUSION

In summary, this study demonstrated that *in vitro* fermentation of KGM and its oligosaccharides by colonic lactic acid bacteria exerted antioxidative effects. These effects of konjac oligosaccharides were generally comparable to those of OF. Consumption of these undigestible carbohydrates may give protection against oxidative stress in the human colon.

## ACKNOWLEDGEMENT

The study is financially supported by the National Science Council of the Republic of China, grant NSC 89-2313-B-040-006.

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