Grape Antioxidant Dietary Fiber Stimulates *Lactobacillus* Growth in Rat Cecum

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**Abstract:** The digesta is a highly active biological system where epithelial cells, microbiota, nondigestible dietary components, and a large number of metabolic products interact. The gut microbiota can be modulated by both endogenous and exogenous substrates. Undigested dietary residues are substrates for colonic microbiota and may influence gut microbial ecology. The objective of this work was to study the capacity of grape antioxidant dietary fiber (GADF), which is rich in polyphenols, to modify the bacterial profile in the cecum of rats. Male adult Wistar rats were fed for 4 wk with diets containing either cellulose or GADF as dietary fiber. The effect of GADF on bacterial growth was evaluated in vitro and on the cecal microbiota of rats using quantitative real time polymerase chain reaction (RT-PCR). The results showed that GADF intake stimulates proliferation of *Lactobacillus* and slightly affects the composition of *Bifidobacterium* species. GADF was also found to have a stimulative effect on *Lactobacillus reuteri* and *Lactobacillus acidophilus* in vitro. These findings suggest that the consumption of a diet rich in plant foods with high dietary fiber and polyphenol content may enhance the gastrointestinal health of the host through microbiota modulation.

**Keywords:** grape antioxidant dietary fiber, *Lactobacillus*, polyphenols

**Practical Application:** Grape antioxidant fiber combines nutritional and physiological properties of dietary fiber and natural antioxidants from grapes. Grape antioxidant fiber could be used as an ingredient for functional foods and as a dietary supplement to increase the intake of dietary fiber and bioactive compounds.

**Introduction**

The beneficial effects of grape consumption—reduction of the risk of cancer, especially gastrointestinal tract tumors (Velurugan and others 2010) and prevention of diseases associated with oxidative stress (Manach and others 2005)—are due in part to their polyphenolic content. Suggested mechanisms whereby grape polyphenols exert beneficial effects include antioxidant, anti-inflammatory, and antiproliferative activities; also induction of cell-cycle arrest and apoptosis (Seeram and others 2009). Grape antioxidant dietary fiber (GADF) is a natural product combining the properties of both dietary fiber and antioxidants in a single material. Physiological properties associated with GADF intake include enhancement of cecal antioxidant status (Goñi and Serrano 2008) and reduction of plasma LDL-cholesterol and blood pressure in hypercholesterolemic subjects (Martin-Carrion and others 2000; Pérez-Jiménez and others 2008b). GADF intake also reduces apoptosis and induces a decline in mucosal thickness, crypt depth, and crypt density in colonic mucosa of Wistar rats, inducing varying degrees of epithelial hypoplasia between the proximal and the distal colon (López-Oliva and others 2006, 2010).

The gut microbiota can be modulated by both endogenous (intestinal mucines, enzymes, gut hormones, mucosa cells) and exogenous substrates. The composition of the diet can influence the profile and metabolic activity of the gut microbial ecology by providing substrates available in the form of undigested dietary residues that are resistant to digestive enzymes and the digestion process (Heavey and Rowland 2004). In this regard, it has recently been proposed that a healthy pattern could be defined on the basis of dietary fiber and bioactive compounds (Saura-Calixto and Goñi 2009).

*Lactobacillus* and *Bifidobacterium* are beneficial gut microorganisms with attributed health properties, mainly inhibition of a wide range of pathogens, improvement of lactose digestion, and stimulation of the immune system through cytokine stimulus (Gotteland and others 2008). Dietary polyphenols are also substrates for colonic microbiota and may inhibit the proliferation of some pathogenic bacteria but also act as promoting factors for growth, proliferation, or survival of beneficial gut bacteria (Hervert-Hernández and Goñi 2011). Several fruit polyphenols have been shown to inhibit the growth and adhesion of *Salmonella* to a human gut cell line and to enhance the proliferation and adhesion of *Lactobacillus rhamnosus* (Parkar and others 2008). In this connection, polyphenols extracted from grape pomace have been found to stimulate beneficial *Lactobacillus acidophilus* growth in vitro (Hervert-Hernández and others 2009). Indeed, the prebiotic effect could be enhanced when a substantial amount...
of polyphenols is associated with dietary fiber. In this context, the objective of the present work was to study the influence of the intake of an antioxidant dietary fiber rich in polyphenols (GADF) on the bacterial profile in the cecum of animals.

Materials and Methods

Samples

GADF is a natural product obtained from red grapes (Cencibel variety, vintage year 2005, La Mancha, Spain). The proximate composition of GADF was previously evaluated and presented in Table 1. GADF was used as a source of dietary fiber in the animal diet. Dietary fiber was determined as indigestible fraction (Saura–Calixto and others 2000). Extraction and measure of extractable polyphenols (EPP) as well as determination of nonextractable polyphenols (NEPP) from GADF were made according to Saura–Calixto and others (2007). The antioxidant capacity (AC) of the extracts was estimated in terms of radical scavenging activity following the procedure described elsewhere (Pérez-Jiménez and others 2008a, Table 1).

Animals and diets

Male Wistar rats with an average body weights of 215 ± 2 g (Harlan Iberica, Spain) were housed in individual metabolic cages and kept in a room at 22 ± 1 °C, 60% humidity and with 12-h light/dark cycles. Two treatment groups were used (n = 10) and were fed either the control diet or the GADF diet for 4 wk. Food and water were freely available. The experimental diets were isocaloric and were manufactured by Dyets Inc. (Bethlehem, Pa., U.S.A.) having identical fiber content (50 g/kg diet) but varying in the type of fiber (cellulose or GADF; Table 1). Animal experimentation was approved by the University committee and followed Directive 86/609 EEC (European Community). At the end of the study, the rats were anesthetized with ketamine (90 mg/kg) and exsanguinated. Cecal content from 10 rats belonging to each group, either control or GADF, was pooled and homogenized with a sterile double-beaded Teflon (Savant Instruments, Farmingdale, N.Y., U.S.A.) homogenizer for 30 s at a speed setting of 8.0. The cecal homogenates were centrifuged at 10,000 × g for 10 min, and the supernatant was collected as cecal fluid for further analysis.

Bacterial strains and cultures

Bifidobacterium longum (ATCC 15707), L. acidophilus (ATCC 4356), and Lactobacillus reuteri (ATCC 23272) were used to evaluate the effects of GADF. These strains, as well as Bacteroides vulgatus (ATCC 8482) and Escherichia coli (ATCC 25922), were used as reference strains for real time polymerase chain reaction (RT-PCR). E. coli and B. vulgatus were grown in Brain Heart Infusion (bioMerieux, Madrid, Spain); Columbia Blood Agar (bioMerieux) was also used for B. vulgatus. MRS Broth and Rogosa Agar (Pronadisa, Madrid, Spain) were used for Lactobacillus spp. and B. longum cultures, supplemented with 0.05% (w/v) l-cysteine for the latter species. Cultures of B. vulgatus and B. longum, and in some cases of L. acidophilus, were incubated in anaerobiosis.

Effects of GADF fiber on bacterial growth

To evaluate the effect of GADF on bacterial growth, Lactobacillus spp. and B. longum strains were grown to OD550 = 1 and diluted 1:200 in fresh medium; 50 μL of these dilutions were inoculated in Falcon 50 mL tubes with 5 mL of the same medium (MRS, supplemented with l-cysteine for B. longum). GADF (2.4, 5, or 20 mg/mL), EPP (1.2 mg/mL), NEPP (2.4 mg/mL), or nothing (control), were added to the corresponding tubes, which were incubated 24 ± 2 h at 37 °C in an orbital shaker (100 rpm), either in an aerobic or anaerobic atmosphere. Growth was measured by plate count in triplicate and plotted as colony forming units (CFU) per mL of culture on a decimal logarithmic scale.

Extraction of bacterial genomic DNA

Cecal content from 10 rats belonging to each group, either control or GADF was pooled and homogenized with a sterile glass rod in 300 μL of PBS. Genomic DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, Calif., U.S.A.) following the recommended protocol with some modifications: samples were previously centrifuged (12,000 g × 5 min at 4 °C) and washed twice with 1 mL of PBS–EDTA, and the homogenization step was done in a FastPrep® Instrument (MP Biomedicals) for 10 s at a speed setting of 5.5. For calibration purposes, DNA of the reference strains of B. vulgatus, B. longum, E. coli, and L. acidophilus was extracted by the method described by Mahenthiralingam and others (1996) using a FastPrep® Instrument. The amount of genomic DNA extracted was estimated by OD260.

Primers for DNA amplification

The primers corresponding to 16S rRNA sequences used in PCR and RT-PCR were: 5′-ACGGATATTCTCCTGTGGT-GC-3′ and 5′-CATGTTCCTCCTCCGCTTGTGC-3′ for B. vulgatus; 5′-GAGGAGCAGTAAAGGAACAGCTTTC-3′ and 5′-GAGGCAGTACCTCTCATCCTCTCC-3′ for Lactobacillus spp. (Fujita and others 2002); 5′-GCTTGGCTGGCTAGAATGAGGC-3′ and 5′-CTGATAGGACGGCCCATCCT-3′ for Bifidobacterium spp. (Gueimonde and others 2004); and 5′-ATGAAAGCCTGCTACAGGAGG-3′ and 5′-GCTTATAGGACGGCCCATCCT-3′ for Bifidobacterium spp.

Table 1–Proximate composition and antioxidant capacity of grape antioxidant dietary fiber

<table>
<thead>
<tr>
<th>Component</th>
<th>Dry matter (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>11.08 ± 0.46</td>
</tr>
<tr>
<td>Fat</td>
<td>7.69 ± 0.49</td>
</tr>
<tr>
<td>Ash</td>
<td>5.25 ± 0.19</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>7.60 ± 3.10</td>
</tr>
<tr>
<td>Soluble dietary fiber</td>
<td>15.53 ± 0.11</td>
</tr>
<tr>
<td>Insoluble dietary fiber</td>
<td>62.07 ± 3.01</td>
</tr>
<tr>
<td>Extractable polyphenols</td>
<td>4.23 ± 0.03</td>
</tr>
<tr>
<td>Nonextractable polyphenols</td>
<td>17.51 ± 0.19</td>
</tr>
<tr>
<td>Antioxidant capacity a (μmol trolox equivalents/g dry matter)</td>
<td>375.50 ± 0.89</td>
</tr>
</tbody>
</table>

aData are expressed as mean ± SD (n ≥ 3).

a Determined as indigestible fraction (Saura–Calixto and others 2000).

b Some polyphenols and resistant protein are included in this value.

c Determined by ABTS method (Pérez-Jiménez and others 2008a).
Quantitative real-time polymerase chain reaction (RT-PCR)

RT-PCR reactions were carried out on MicroAmp® Fast Optical 96-Well Reaction Plates using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, Calif., U.S.A.). Each reaction contained 5 μL of diluted DNA, 1 μL of a 5 μM solution of each primer, and 10 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 20 μL, and each DNA dilution (10^{-1} to 10^{-7}) was duplicated. The cycle threshold of each sample was compared to a standard curve made by diluting (10^{-3} to 10^{-7}) genomic DNA obtained from B. vulgatus, L. acidophilus, B. longum, and E. coli. These bacteria were enumerated by plate count before DNA extraction. Specificity of the RT-PCR reactions, melt curve analysis was determined after amplification by slow cooling from 95 to 60 °C and reheating to 95 °C in the same 7900HT equipment. The fragments detected by RT-PCR with Bifidobacterium spp. primers were sequenced by PCR amplification with the same primers, using the ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction Kit in an automated ABI PRISM 377DNA Sequencer (Applied Biosystems). Similarities with 16S rRNA sequences stored in Gen-Bank and EMBL databases were searched with the BLAST software (www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

Results were expressed as mean and standard deviations, as detailed in the legends of Figure 1 and 2. One-way ANOVA with the post hoc Bonferroni test was used to find differences due to the type of dietary fiber within the same type of organism and condition (aerobic or anaerobic; Figure 1A and 1B). Differences in the in vivo assay were assessed by an unpaired Student’s t-test (Figure 2). In both cases differences were considered significant if P < 0.05. The statistical analyses were conducted using SPSS 15.1 for Windows (SPSS, Chicago, Ill., U.S.A.)

Results and Discussion

Bacterial counts from aerobic and anaerobic cultures in the absence or presence of GADF are shown in Figure 1. There was a significant increase (P < 0.001) of the growth of Lactobacillus spp. in the presence of GADF at 2.4 mg/mL (about 2 log units, Figure 1A). Higher concentrations of GADF (5 and 20 mg/mL) were tested with L. acidophilus, but the increase of growth was not significant with respect to the lowest concentration used, suggesting a saturation dynamics. Fractions EPP and NEPP also stimulated the growth of both Lactobacillus species (P < 0.001), NEPP being slightly more efficient than EPP (P < 0.001). However, whereas the growth increase of L. reuteri by these fractions was more or less similar to the induced with GADF, in the case of L. acidophilus the stimulation was clearly lower.

GADF did not significantly affect growth of B. longum (Figure 1B). To rule out a possible effect of the anaerobic conditions in which B. longum was cultivated, the assay was repeated with L. acidophilus grown in anaerobiosis, with comparable results to those obtained in aerobic conditions. Again, the addition of GADF at a higher concentration (20 mg/mL) produced no significant increase in the growth of either of these bacteria (Figure 1B).

Next, the effect of GADF in physiological conditions was explored. Two groups of rats were fed with diets containing either cellulose or GADF (50 g/kg, Table 1) and the cecal microbiota were analyzed by RT-PCR, which has proven to be a reliable method for quantifying Lactobacillus spp. and Bifidobacterium spp. in rat fecal samples (Delroiase and others 2008). This assay was complemented by using specific primers to quantify E. coli and B. vulgatus (Bej and others 1991). The primers designed for RT-PCR were first checked in a normal PCR amplification with DNA from cultures of the reference strains of B. vulgatus, B. longum, L. acidophilus, and E. coli, and also with DNA extracted from the cecal samples. Single bands of the expected size were amplified, and melt curve analysis was used to determine after amplification by slow cooling from 95 to 60 °C and reheating to 95 °C in the same 7900HT equipment. The fragments detected by RT-PCR with Bifidobacterium spp. primers were sequenced by PCR amplification with the same primers, using the ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction Kit in an automated ABI PRISM 377DNA Sequencer (Applied Biosystems). Similarities with 16S rRNA sequences stored in Gen-Bank and EMBL databases were searched with the BLAST software (www.ncbi.nlm.nih.gov/BLAST).

Figure 1—Effect of grape antioxidant dietary fiber (GADF) on bacterial growth, measured by plate count and expressed in decimal logs of CFU per mL of culture. GADF, EPP (extractable polyphenols), NEPP (nonextractable polyphenols), or nothing (control) were added to the cultures at the indicated final concentrations (in mg/mL) and were incubated 24 ± 2 h at 37 °C in an orbital shaker (100 rpm) in aerobic (Panel A) or anaerobic (Panel B) conditions. Error bars represent the SD of the triplicate bacterial counts of 2 assays (for Lactobacillus spp.) or 3 assays (for B. longum). *: P < 0.001; +: P < 0.01; #: P ≤ 0.02 with regard to control; **: P < 0.001, +*: P ≤ 0.02 with regard to GADF 2.4 mg/mL; ¥: P < 0.001, ¥¥: P ≤ 0.02 with regard to EPP.

Figure 2—Quantification of cecal bacteria by RT-PCR in the group of rats fed with grape antioxidant dietary fiber (GADF) and in the control group. Results are expressed in decimal logs of CFU per g of cecal content. Data represent the mean of the results obtained in 2 different PCR reactions, each performed with 3 decimal dilutions of the extracted DNA; in the case of Bifidobacterium spp., 2 independent DNA extractions were analyzed. *: P < 0.001.

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detected in each case (data not shown). Results of the RT-PCR quantification in both groups of rats are summarized in Figure 2. Rats fed with GADF showed a roughly one-log increase of *Lactobacillus* spp. with respect to the control group (2.4 ± 0.5 compared with 3.52 ± 10^4). On the other hand, populations of *Bifidobacterium* decreased by less than 1 log in the GADF-fed group (4.4 × 10^2 compared with 1.4 × 10^3). Changes in *E. coli* and *Bacteroides* counts were not significant.

Our major finding was that the increase in the lactobacilli population in vivo clearly correlates with the observed effects of GADF on *L. reuteri* and *L. acidophilus* in vitro. This is important in that a gut microbial profile in which beneficial bacteria predominant over detrimental bacteria may be beneficial to the health of the host, promote a beneficial immune function (Salmínen and Isolauri 2006), and help to prevent gastrointestinal disorders. Our results are supported by a study on the administration of red wine polyphenols to rats fed with a high-fat diet (Dolara and others 2005), which reported that *Lactobacillus* and *Bifidobacterium* were more common in feces of polyphenol-treated rats than in the control. The predominance of *Lactobacillus* (but not of *Bifidobacterium*) over Clostridium in the treated group was significant. Other results reported by Tzounis and others (2008) in a fecal batch culture with colonic microbiota fermenting tea polyphenols showed an increment of *Lactobacillus*, *Bifidobacterium*, and *E. coli* during fermentation of (−)-catechin. Changes in these populations were more complex during fermentation of (−)-epicatechin, showing opposite behavior of both *Lactobacillus* and *E. coli* depending on the polyphenol concentration, and a very slight decline of *Bifidobacterium*.

The results for GADF effects on *Bifidobacterium*, were less clear. There was a slight but not significant (P > 0.05) increase of growth in vitro and a decrease in the population in vivo. Varying effects of GADF on the different *Bifidobacterium* species present in the colonic microbiota that may be detectable by RT-PCR, cannot be ruled out, and so this possibility was investigated. Therefore, thermal dissociation curves were performed post-RT-PCR to verify the uniformity of the amplification products in both the GADF and control groups. These dissociation curves were uniform for *Bacteroides*, *Lactobacillus*, and *E. coli*, but in the case of *Bifidobacterium* the peak of the dissociation curves of the amplification products from the control group (85.1 °C) was not coincident with that of the GADF group (82.7 °C), which was coincident with the peak detected for the reference *B. longum* strain (82.9 °C).

To clarify this discrepancy, the DNA sample from the control group was amplified and sequenced with the same 16S rRNA *Bifidobacterium* spp. primers. This sequence gave the highest identity (95%) with *Bifidobacterium* pseudolongum (accession nr AB107570) accurate enough, and so we can only conclude that RT-PCR detected different species in each control and GADF rat group. This finding supports the hypothesis that the diet can have specific effects on different *Bifidobacterium* species and may cause changes in the predominance of these species, detectable by RT-PCR. Although we cannot rule out a different starting composition of the microbiota in each group, this possibility is not likely given that each group was composed of 10 randomly chosen individuals. In any case, this finding shows that a much more thorough analysis in vitro and in vivo of the GADF activity over several species of this genus is required.

**Conclusions**

GADF intake produces a significant increase of beneficial *Lactobacillus* but not of *Bifidobacterium* in the cecum of rats, confirming the stimulative effect on proliferation of 2 species of *Lactobacillus* observed in vitro. This study supports the hypothesis that the consumption of a diet rich in plant foods with high dietary fiber and polyphenol content may help to enhance the gastrointestinal health of the host by promoting a beneficial microbiota profile.

**References**


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