IN VITRO AND ANIMAL STUDIES

Potential fat-lowering and prebiotic effects of enzymatically treated okara in high-cholesterol-fed Wistar rats

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ABSTRACT
This study evaluates the effect of the lipid profile on serum, liver and faeces, and the potential prebiotic effect of diets supplemented with enzymatically treated okara (okaraET) in high-cholesterol fed Wistar rats. Triglyceride levels were significantly reduced in the serum \((p < 0.01)\) and liver \((p < 0.01)\) of okaraET treated rats. Total lipids, triglycerides and bile acids were significantly higher \((p < 0.001)\) in the faeces of rats fed the okaraET diet. The pH of faecal contents from treated okaraET rats was lower \((p < 0.001)\), probably due to the significantly higher \((p < 0.001)\) production of short-chain fatty acids (SCFA). OkaraET, therefore, reduced triglycerides in serum and liver, and increased the excretion of total lipids, triglycerides and bile acids, improving the lipid profile in rats fed with high-cholesterol diets. OkaraET fibre can improve intestinal transit by increasing faecal bulk. The decreased pH and increased SCFA production indicated that okaraET fibre fermentation occurred, suggesting a potential prebiotic effect.

ARTICLE HISTORY
Received 14 January 2016
Revised 6 June 2016
Accepted 7 June 2016
Published online 27 June 2016

KEYWORDS
Dietary fibre; fermentability; hypocholesterolaemic effect; okara; prebiotic

Introduction
Soybeans are a source of functional ingredients that are of interest from a nutritional standpoint due to their potential health effects of their dietary fibre and protein contents, especially under hyperlipidaemic and atherogenic conditions. Soybeans and soy foods are widely promoted and consumed, as they are assumed to confer health benefits (Mateos-Aparicio et al. 2008, 2013). Soybeans are commonly processed to obtain protein isolates and other end-products such as soy-milk and tofu. Large quantities of a fibre-rich residue called okara are removed during this process. 1.4–1.8 kg of fresh okara is obtained from approximately 1 kg of dry soybean seeds for soy beverage production. Okara has a high total dietary fibre content of 54–55%, mainly insoluble dietary fibre (IDF) (Mateos-Aparicio et al. 2010a). The functionality of okara has previously been reported due to its hypocholesterolaemic and hypolipidemiatic effects (Villanueva et al. 2011) and antioxidant activity (Mateos-Aparicio et al. 2010b). Okara may be useful as a weight-loss dietary supplement (Préstamo et al. 2007), and may protect the gut environment in terms of antioxidant status and prebiotic effect (Jiménez-Escrig et al. 2008).

It is generally accepted that non-digestible dietary carbohydrates are the main substrates available for fermentation by bacteria in the human colon, and when this fermentation is produced by selective bacteria – which have a beneficial effect on the gut microbiota – they are considered prebiotics (Corzo et al. 2015). The intake of soluble viscous fibres, in combination with other dietary changes such as reduced fat consumption, is effective at decreasing cholesterol levels (Gray 2006). It is, therefore, beneficial to enhance okara functionality by increasing soluble fibre. Several strategies have been studied by our research group. First, the effect of combined hydration, mild temperature and high hydrostatic pressure treatment increases the percentage of the soluble fraction in okara (Mateos-Aparicio et al. 2010c). Enzymatic treatment with Ultraflo L® to enhance the ratio of soluble:insoluble fibre was recently used to obtain enzymatically treated okara (okaraET) and improve its in vitro prebiotic effect (Villanueva-Suárez et al. 2013). The aim of this study was to evaluate the influence of okaraET intake...
for 4 weeks on lipid metabolism and the health-promoting effects in the gut in hypercholesterolaemic Wistar rats.

Materials and methods

Sample preparation

Okara, a by-product of soymilk production, was supplied by the company Too Fu Ya, S.L. (Arganda del Rey, Spain). The okara was enzymatically treated with a commercial enzyme, Ultraflo L\textsuperscript{V} (Novozymes, Bagsvaerd, Denmark) in order to obtain okara\textsuperscript{ET}. This commercial enzyme present β-1,4-glucanase activity mainly and also cellulase, xylanase, pentosanase and arabanase activity. Dry okara was mixed with water (1:3 w/w) and autoclaved. The enzymatic hydrolysis was performed by adding 0.05% of Ultraflo L\textsuperscript{V} (Villanueva-Suárez et al. 2013). The composition of this product compared with the untreated material is showed in Table 1.

Animals and diets

Wistar Hannover rats (4 weeks old) were housed in cages in a room with controlled light (12 h, 08:00–20:00), temperature (22 ± 1 °C) and unrestricted access to food and water. The animals were maintained in accordance with the guidelines from the registered laboratory (N° 28079-15ABC-M, Madrid, Spain). The study protocol was approved by the Animal Experimentation Committee of the Universidad Complutense de Madrid, in accordance with RD 53/2013. The 24 animals were fed a commercial chow diet for 1 week in order to acclimatise them. The rats were randomised (n = 12) and fed high-cholesterol diets for 4 weeks. The supplemented diet was prepared with 20% fibre from the okara\textsuperscript{ET}. The diets were adjusted to obtain a similar composition of protein, fat, carbohydrates, minerals and vitamins (Table 2).

During the experiment, the rats and feed were weighed weekly. The faeces were collected, weighed, freeze-dried and stored until analysis at the end of each week. At the end of the experimental period, the rats were anesthetised with isoflurane and blood samples were collected by cardiac puncture. The organs (heart, liver, spleen, kidneys and caecum) were removed and weighed.

Determination of serum parameters

Glucose, uric acid, albumin, cholesterol and triglycerides from the plasma were analysed in the Autoanalyzer Cobas Integra 400 plus (Roche, Basel, Switzerland). High-density lipoprotein (HDL) and low-density lipoprotein (LDL) were measured using an enzymatic method (Spinreact, Girona, Spain). LDL cholesterol was calculated as the difference between total and HDL cholesterol (Villanueva et al. 2014).

Determination of hepatic and faecal lipids

Total fat was extracted from the freeze-dried livers and faeces with petroleum ether in a Soxtec System HT extractor (1043 Extraction Unit). Total cholesterol and triglycerides were determined using specific enzymatic colorimetric methods (Spinreact) (Villanueva et al. 2014). Cholesterol esters were hydrolysed with cholesterol esterase. Both hydrolysed and free cholesterol were oxidised to hydrogen peroxide, which reacts with phenol and 4-aminophenazone and the resulting colour was measured spectrophotometrically. Triglycerides were determined by a colorimetric reaction with 4-aminoantipyrine, p-chlorophenol, catalysed by peroxidase, and the absorbance was measured at 505 nm (Villanueva et al. 2014). The bile acids were extracted with 96% ethanol for 24 h from faeces and analysed by an enzymatic colorimetric method (Materlab, Madrid, Spain) (Villanueva et al. 2014).

| Ingredients | Control diet\textsuperscript{a} | Supplemented diet\textsuperscript{b} |
|-------------|-----------------|-----------------|
| Purified diet 210 | 557 | 557 |
| Okara\textsuperscript{ET} | – | 431 |
| Cholesterol | 10 | 10 |
| Cholic acid | 2 | 2 |
| Casein | 160 | – |
| Corn oil | 40 | – |
| Glucose | 31 | – |
| Cellulose | 200 | – |
| Energy (Kcal/Kg) | 3547 | 3589 |
| (KJ/Kg) | 14,850 | 15,026 |

\textsuperscript{a}Control diet: AIN-210 purified rodent diet with 20% microcrystalline cellulose.

\textsuperscript{b}Supplemented diet: AIN-210 purified rodent diet with 20% fibre from the okara\textsuperscript{ET}.

Table 1. The proximal composition of okara\textsuperscript{ET} and untreated okara (g/100 g).

| Protein | Okara | Okara\textsuperscript{ET} | p Value |
|---------|-------|-----------------|---------|
| 32.3 ± 1.2 | 31.5 ± 1.1 | ns |
| Fat | 11.9 ± 0.2 | 9.1 ± 0.1 | * |
| Sugars | 3.3 ± 0.5 | 6.5 ± 0.9 | *** |
| Oligosaccharides | 1.2 ± 0.5 | 5.1 ± 1.1 | *** |
| Ash | 2.6 ± 0.3 | 4.0 ± 0.5 | *** |
| Total DF | 48.6 ± 2.5 | 43.8 ± 1.9 | * |
| IDF | 44.5 ± 2.4 | 33.7 ± 1.4 | *** |
| SDF | 4.1 ± 0.6 | 10.1 ± 1.1 | *** |
| IDF/SDF | 10.7 | 3.3 | – |

DF: dietary fibre; IDF: insoluble dietary fibre; SDF: soluble dietary fibre.
ANOVA *p < 0.05; ***p < 0.001; ns: non-significant.
Measurement of pH and short-chain fatty acids analysis in caecal content

The content of caecum was aseptically collected for the determination of pH and short-chain fatty acids (SCFA). A caecal portion was diluted at 1:3 in Milli-Q deionised water (Millipore Iberica, Madrid, Spain) in order to measure the pH using a microelectrode Crison micro pH 2001 (Barcelona, Spain). The diluted sample was stored at −80 °C until SCFA analysis. After thawed, samples were centrifuged at 9000 g for 15 min at 4 °C, and the supernatents were utilised for gas–liquid chromatography (Perkin-Elmer Autosystem, Waltham, MA), using 4-methyl valeric acid as the internal standard (Jiménez-Escrig et al. 2008).

Statistical analysis

Values are expressed as mean ± standard deviation. Data were analysed using one-way analysis of variance (ANOVA) conducted with Statgraphics Plus version 5.1 (Warrenton, VA).

Results

Effect on food intake and body weight

Daily observation of all animals revealed that the diets were well accepted and that the animals remained seemingly healthy during the experiment. Dietary intake differed significantly (p < 0.01) between rats fed the supplemented diets with okara ET and control rats. These results had a positive effect on feed efficiency (p < 0.05) (Table 3). The body weight of the rats increased progressively in both groups during the 4 weeks of the experiment. Animal weight gains were 19% (with the okara ET diet) and 14% (with the control diet) (Figure S1), but no significant differences were found between both groups due to individual variability (Table 3). There were no significant differences in the weight of the organs (heart, spleen, kidneys and liver) and the longitudinal measure of the gastrointestinal tract. A visual inspection of the organs did not show macroscopic alterations in either group of rats.

Effect on serum lipid profile and other parameters

Glucose, albumin and uric acid were not significantly affected by diet (Table 4). A slight decrease in total cholesterol and LDL + VLDL and increase in HDL concentrations was observed in rats fed with okara ET, although no significant changes were detected after 4 weeks of the experiment. However, the supplementation with okara ET significantly decreased (p < 0.01) triglycerides (25.5%).

Effect on liver lipid profile

Table 5 shows that triglycerides content in the liver was significantly lower (p < 0.01) in the okara ET fed-group with a decrease of 22.4% in comparison with the control animals, suggesting its hypolipidaemic activity. Total cholesterol, esterified cholesterol and free cholesterol did not differ significantly in comparison with the control.

Effect on faecal excretion

The okara ET diet increased faecal excretion (p < 0.001) throughout the entire experiment. In fact, treated rats

| Table 3. Effect of a diet supplemented with okara ET in the initial and final body weight, body weight gain, food intake and feed efficiency in Wistar rats. |
|------------------|------------------|------------------|
| Control group a  | Treated group b   | p Value          |
| Initial body weight (g) | 217.4 ± 7.5      | 217.0 ± 7.7      | ns              |
| Final body weight (g)    | 248.1 ± 11.4     | 258.0 ± 10.2     | ns              |
| Body weight gain (g/day) | 0.9 ± 0.2        | 1.2 ± 0.2        | ns              |
| Food intake (g/day)      | 14.9 ± 0.5       | 13.7 ± 0.6       | **              |
| Feed efficiency c        | 0.059            | 0.085            | *               |

aControl group: animal group fed with control diet.
bSupplemented group: animal group fed with supplemented diet (okara ET).
cFeed efficiency = body weight gain/food intake.
Values are expressed as mean ± SD (n = 12). ANOVA *p < 0.05; **p < 0.01; ns: non-significant.

Table 4. Effect of a hypercholesterolaemic diet supplemented with okara ET on some plasma parameters in Wistar rats.

|                      | Control group a | Treated group b | p Value |
|----------------------|-----------------|-----------------|---------|
| Glucose (mg/dl)      | 155.7 ± 9.6     | 155.4 ± 13.4    | ns      |
| Albumin (g/dl)       | 3.8 ± 0.5       | 3.8 ± 0.4       | ns      |
| Uric acid (mg/dl)    | 1.2 ± 0.4       | 1.2 ± 0.6       | ns      |
| Triglycerides (mg/dl)| 87.8 ± 21.9     | 65.4 ± 10.1     | **      |
| Cholesterol (mg/dl)  | 234.2 ± 28.4    | 230.9 ± 35.4    | ns      |
| HDL (mg/dl)          | 64.4 ± 10.6     | 70.9 ± 5.3      | ns      |
| LDL + VLDL (mg/dl)   | 170.0 ± 32.5    | 160.0 ± 31.9    | ns      |
| Atherogenic Index d  | 3.74 ± 0.89     | 3.32 ± 0.55     | **      |

aControl group: animal group fed with control diet.
bSupplemented group: animal group fed with supplemented diet (okara ET).
cAtherogenic Index: total cholesterol (mg/dl)/HDL (mg/dl).
dValues are expressed as mean ± SD (n = 12). ANOVA **p < 0.01; ns: non-significant.

Table 5. Effect of a hypercholesterolaemic diet supplemented with okara ET on concentrations of lipids in the liver (expressed as dry matter).

|                      | Control group a | Treated group b | p Value |
|----------------------|-----------------|-----------------|---------|
| Total lipids (mg/g)  | 414.4 ± 21.0    | 401.9 ± 26.7    | ns      |
| Triglycerides (mg/g) | 104.3 ± 9.8     | 80.9 ± 4.7      | **      |
| Total cholesterol (mg/g) | 74.7 ± 10.3     | 72.2 ± 11.3     | ns      |
| Free cholesterol (mg/g) | 19.8 ± 8.8     | 16.4 ± 4.9      | ns      |
| Esterified cholesterol (mg/g)| 54.8 ± 16.7 | 55.8 ± 8.6 | ns      |

aControl group: animal group fed with control diet.
bSupplemented group: animal group fed with supplemented diet (okara ET).
Values are expressed as mean ± SD (n = 12). ANOVA **p < 0.01; ns: non-significant.
excreted 28.6% more than the controls during the final week of the experiment. The daily faecal weight was higher for the okaraET-fed group than for the controls throughout the experiment (Figure S2). This was attributed mainly to the higher ($p < 0.001$) faecal moisture in the okaraET-fed group ($67.9 ± 5.3$ g/100 g) as compared to the control group ($42.3 ± 3.5$ g/100 g) and to the greater ($p < 0.001$) amount of fat (Table 6). The faecal excretion of total lipids, triglycerides and bile acids is higher ($p < 0.001$) in okaraET-fed rats, approximately 23%, 67% and 49%, respectively.

**Okara fermentation: effect on intestine and pH and short chain fatty acids in the caecum**

The entire (control: 105.64 ± 8.25 cm; treated: 117.30 ± 4.22 cm) and small (control: 10.55 ± 2.73 cm; treated: 15.50 ± 3.75 cm) intestines were enlarged ($p < 0.05$). A high faecal excretion and higher caecum weights ($p < 0.05$) were also observed in okaraET-fed rats (7.24 ± 1.17 g) compared to the controls (2.46 ± 0.51 g). The okaraET-fed rats caecal (pH 5.5) was lower ($p < 0.001$) than in control rats (pH 7.1). The fermentation of okaraET fibre produced more than twice the total SCFA than that of the control group (Table 7). Acetate was the main SCFA produced followed by isobutyric, butyric and propionic; however, valeric acid was in smaller proportion. All SCFA values were found to be higher ($p < 0.001$) in the treated group except isovaleric acid (Table 7).

**Table 6.** Effect of a hypercholesterolaemic diet supplemented with okaraET on concentrations of lipids, triglycerides, cholesterol and bile acids in faeces (expressed as dry matter).

|                     | Control group | Treated group | $p$ Value |
|---------------------|---------------|---------------|-----------|
| Total lipids (mg/g) | 36.6 ± 1.8    | 47.3 ± 3.9    | ***       |
| Triglycerides (mg/g)| 5.9 ± 0.9     | 18.0 ± 0.4    | ***       |
| Cholesterol (mg/g)  | 19.9 ± 3.6    | 23.2 ± 3.5    | ns        |
| Bile acids (μmol/g)| 6.2 ± 1.3     | 12.1 ± 1.8    | ***       |

*Control group: animal group fed with control diet.
Supplemented group: animal group fed with supplemented diet (okaraET).

Values are expressed as mean ± SD ($n = 12$). ANOVA ***$p < 0.001$; ns: non-significant.

**Table 7.** pH and short chain fatty acids (mmol/g) in the Wistar rat caecal contents.

|                     | Control group | Treated group | $p$ Value |
|---------------------|---------------|---------------|-----------|
| pH                  | 7.1 ± 0.1     | 5.5 ± 0.2     | ***       |
| Acetic acid         | 140.1 ± 4.9   | 319.5 ± 55.2  | ***       |
| Propionic acid      | 12.0 ± 4.5    | 71.4 ± 28.5   | ***       |
| Isobutyric acid     | 37.3 ± 6.7    | 86.8 ± 17.0   | ***       |
| Butyric acid        | 31.5 ± 8.5    | 76.9 ± 26.6   | ***       |
| Isovaleric acid     | 5.4 ± 1.2     | 7.1 ± 3.1     | ns        |
| Valeric acid        | 2.9 ± 0.7     | 6.5 ± 2.0     | ***       |
| Total SCFA          | 229.2 ± 37.0  | 568.2 ± 56.5  | ***       |

*Control group: animal group fed with control diet.
Supplemented group: animal group fed with supplemented diet (okaraET).

Values are expressed as mean ± SD ($n = 12$). ANOVA ***$p < 0.001$; ns: non-significant.

**Discussion**

The okaraET has a higher content of oligosaccharides and soluble dietary fibre (SDF), at the cost of the hydrolysis of the IDF. The IDF/SDF ratio of okaraET is more than three times lower than in untreated okara (Villanueva-Suárez et al. 2013).

The serum lipid profile of okaraET-fed rats showed a significant decrease in TG and a tendency to decrease LDL+VLDL and increase HDL. Jiménez-Escrig et al. (2008) found no differences in the plasma triglycerides of rats fed diets enriched with a lower proportion of okara (10%) over a shorter time (3 weeks). Matsumoto et al. (2007) concluded that a diet supplemented with 40% okara significantly reduces total cholesterol and LDL-C in mice after a 10-week trial period. Villanueva et al. (2011) reported quite similar effects in plasma parameters (TG and LDL-C) to the findings of the present study in Syrian hamsters supplemented with 20% okara fibre. Bedani et al. (2015) showed that the consumption of synbiotic fermented soy product supplemented with okara over 8 weeks significantly decreased LDL-C and improved the LDL-C/HDL-C ratio, and had a preventive effect on cardiovascular disease risk markers in healthy men. The TG amount was also significantly lower in the livers of okaraET-fed rats. Villanueva et al. (2011) found a significant decrease in triglycerides and a reduction in total cholesterol and esterified cholesterol in the livers of Syrian hamsters fed with 20% okara fibre. However, the diets supplemented with 13% okara fibre tested by these authors only revealed a decrease in total hepatic lipids. Matsumoto et al. (2007) reported that okara intake dose-dependently suppresses the development of body weight and epidymal white adipose tissue, and prevents an increase in plasma lipids and hepatic steatosis. Regarding the faecal excretion, it was increased as moisture and fat was significantly greater for rats treated with the supplemented okaraET diet as in the results reported by Villanueva et al. (2011) in Syrian hamsters fed 20% okara fibre. This fat excretion is expected due to the increased swelling and water and oil retention capacities presented in okaraET (Villanueva-Suárez et al. 2013) as compared to untreated okara (Mateos-Aparicio et al. 2010d) since the okaraET presents more soluble fibre. However, this effect does not seem to be related with the dietary fibre co-passengers, and it is strongly dependent on the soluble fibre viscosity itself; this property hinders the micelle formation that is essential for fat absorption (Lairon et al. 2007). Bile acid excretion was significantly increased, which could indicate an increased bile acid synthesis. Therefore, the TG decrease found in
plasma and liver seem to be related with the greater excretion of lipids in faeces. Despite the fat excretion, there was not body weight loss as Préstamo et al. (2007) observe. The higher sugars content in okara ET compared to untreated okara could provide energy.

Okara ET fibre enhanced the excretion of faecal bile acids and may increase bile acid biosynthesis, as okara intake causes a dose-dependent increase in the activity of 7α-hydroxylase, a key enzyme in the biosynthesis of bile acids (Matsumoto et al. 2007). Okara ET has a notable amount of soluble fibre and oligosaccharides that enhance colon fermentation compared to untreated okara (Mateos-Aparicio et al. 2010d; Villanueva-Suárez et al. 2013). Fermentation involves a proliferation of bacterial biomass that further contributes to faecal bulk and stool consistency. Consistency is also influenced by the high moisture found in the faeces of treated rats, due to the reduction in colonic water resorption (Yasmin et al. 2015). This colonic fermentation produced a significant amount of SCFA, which may be directly responsible for a reduction in intraluminal pH, or may increase the bacteria that potentially cause the decrease (Busche & von Engelhardt 2007).

Acidification could be responsible for the insolubility of the bile acids by decreasing their passive diffusion absorption (Favier et al. 1997). Constituents other than fermentable polysaccharides may modify the pH, i.e. resistant proteins can increase pH and improve the productivity of total SCFA (Cummings et al. 1987). Propionic acid also increased (approximately five times higher). This SCFA is responsible for a decrease in fatty acid biosynthesis and may act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A, which is the main enzyme implicated in the endogenous synthesis of cholesterol (Al-Lahham et al. 2010). The hypocholesterolaemic effect was not observed in our study although there was major excretion of total lipids and bile acids as mentioned above. In the light of these results, a cholesterol decrease could possibly be observed if the assay were prolonged or if the okara amount was increased, as described by Bedani et al. (2015) and Matsumoto et al. (2007), respectively.

Conclusion

The elimination of lipids through faeces and the triglycerides decrease in rat sera and livers highlight the fat-lowering effects of okara ET. This role has been mainly attributed to the increase of the SDF and oligosaccharides contents in okara ET compared to untreated okara. The rise faecal excretion and the softening of the faeces can facilitate the evacuation. The okara ET fermentation produced a great SCFA content and reveals the positive activity in the intestine and the potential prebiotic effect. Therefore, these results suggest that okara ET could be a source of dietary fibre having potentially prebiotic properties and beneficial effects on cardiovascular diseases, thus being a value-added ingredient in other food products.

Acknowledgements

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practise at which the studies were conducted.

Disclosure statement

All authors have no conflict of interest to report regarding the work presented in this manuscript.

Funding information

This research was supported by the Ministry of Science and Technology (Spain), Project AGL 2005-02447 and by Universidad Complutense de Madrid, Project GR3/14.

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