POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2009, Vol. 59, No. 4, pp. 295-298

INFLUENCE OF PLANT EXTRACTS ADDITION ON THE ANTIOXIDATIVE PROPERTIES OF PRODUCTS OBTAINED FROM GREEN LENTIL SEEDS DURING *IN VITRO* DIGESTION PROCESS

Małgorzata Gumienna, Małgorzata Lasik, Zbigniew Czarnecki

Institute of Food Technology of Plant Origin, Department of Fermentation and Biosynthesis, Poznań University of Life Sciences, Poland

Key words: intestinal microflora, polyphenols, antioxidative activity, green lentil seeds

The aim of the presented research was to determine the activity and interactions of the human intestinal microflora with biologically-active compounds as well as the degree of their *in vitro* survivability in the applied model of the gastrointestinal tract. Products (pasta) from green lentil seeds were obtained after thermoplastic treatment with the addition of plant extracts of garlic, onion and oregano (concentration of water extracts was 50 mg/mL). The highest antioxidative activity (32.43 mg Trolox/g) and content of phenolic compounds (3.21 mg/g) after the digestion process were determined for pasta from green lentil with the oregano extracts. Growth of the investigated bacteria (*Enterobacteriaceae*, *Lactobacillus, Enterococcus,* and *Bifidobacterium* genera) was affected by the digested noodles from green lentil with the plant extracts, especially in the case of the *Enterococcus* and *Lactobacillus* species. The bacterial count after digestion in the large intestine was maintained at a level of 10^8 cfu/mL, whereas the noodles without the addition of plant extracts also stimulated microflora growth. As a result, the count of colonic bacteria was found to reach 10^9-10^{10} cfu/mL.

INTRODUCTION

After birth, the human gastrointestinal tract becomes readily colonized with microorganisms originating from the mother and the environment. The composition of this microbial community is relatively simple in infants but very complex in adults. In has been estimated that more than 400 different bacterial species contribute to an estimated total number of 10^{13} bacterial cells resident in the human gastrointestinal tract. Although there are certain similarities with respect to the general makeup of the bacterial community, it has to be emphasized that the composition of the intestinal microbiota is highly variable between subjects and, when analysed in detail, actually unique for each individual. The density of bacterial cells in the gastrointestinal contents increases by up to 11 orders of magnitude from levels of 10^1 to 10^3 colony forming units (cfu) per g of contents in the stomach to 10^{12} cfu per g of contents in the distal colon [Blaut *et al*., 2003; Konishi & Kobayashi, 2004].

Legumes have been cultivated by man since time immemorial. They play an important role in human and animal nutrition because of their high content of protein. However, these plants – apart from their high content of nutrients – also contain such bioactive substances as antioxidants which comprise phenolic compounds, carotenoids and vitamins C and E [Frias *et al*., 2005]. Amarowicz & Raab [1997] demonstrated that the antioxidative efficiency of the leguminous seeds (pea, bean, lentil, faba bean, broad bean) extracts varied markedly and did not dependent upon their content of phenolic compounds. In the research upon the lentil seeds Amarowicz *et al*. [2003] obtained four fractions TLC analysis. Fractions III and IV showed the strongest antioxidant activity and the fractions IV had the highest concentration of tannins.

Many antioxidants – contained, primarily in spices and herbs – such as: oregano, thyme, marjoram or rosemary, are hardly applicable due to their very characteristic aroma which they introduce into food. That is why it is important to bring in new products which will take advantage not only of the antioxidative potential of spices but, equally importantly, also of their sensory properties [Pellegrini *et al*., 2000; Mc Carthy *et al*., 2001]. Unfortunately, still little data is available on the behavior of these compounds in the man's gastrointestinal tracts, in particular, on their transformations under the influence of the intestinal microflora as well as their toxicity.

The objective of the presented study was to determine the activity and interactions of the human intestinal microflora with biologically-active compounds as well as the degree of their *in vitro* survivability in the applied model of the gastrointestinal tract.

MATERIALS AND METHODS

Material

The experimental material comprised seeds of green lentil obtained from the harvest of 2006. The following materials were used in experiments carried out in the developed model

Author's address for correspondence: Małgorzata Gumienna, Institute of Food Technology of Plant Origin, Department of Fermentation and Biosynthesis, Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland; tel.: (48 61) 84 87 267; fax: (48 61) 84 87 314; e-mail: gumienna@au.poznan.pl

of the *in vitro* gastrointestinal tract: plant extracts of garlic (*Allium sativum* L), onion (*Allium cepa* L.) and oregano (*Origanum vulgare* L.), products (pasta) from green lentil seeds after thermoplastic treatment with or without the addition of plant extracts (reference sample). Plant materials were suspended in water in the concentration of 50 mg/mL and extracted by shaking for 30 min at 150 rpm. The extracts were sterilized by micro-filtration using 0.22 μm Millipore filters. The pasta products were obtained from the seed meal and wheat flour – semolina mixed at the ratio of 1:1 and water plant extract. The obtained water plant extracts were added into the grinded material in the amount of 0.3 mL/g to achieve the final moisture of 33%. Afterwards, the thermoplastic treatment of this material was performed. The parameters of the thermoplastic treatment were selected on the basis of our own investigations [Gumienna *et al*., 2007].

Conditions of the *in vitro* digestion process

The *in vitro* digestion was conducted in a glass bioreactor equipped in 4 inlets allowing the introduction of the pH electrode, programming of the active acidity, dosage of biochemical agents and appropriate media as well as collection of analytical samples. Samples for the *in vitro* digestion process were prepared by taking 20 g of products and dissolving them in tap water to the volume of 200 mL.

The conditions of the "digestion" process in the bioreactor were designed in such a way as to comprise the following stages of the model: the "stomach", the "small intestine" and the "large intestine". The parameters of the digestion process were selected on the basis of our own investigations [Gumienna *et al*., 2007] but also taking into consideration studies carried out by Gil-Izquierdo *et al*. [2001] and Knarreborg *et al*. [2002]. In order to control the influence of the conditions prevailing in the gastrointestinal tract on the growth of microorganisms, control inoculations were made after 2 h from the moment (pH 7.4, small intestine) of introducing the microorganisms into the environment and at the moment of termination of the digestion process (after 21 h). Intestinal microflora isolated from faeces of a mature person was introduced into the experimental model. The determined groups of microorganisms included: *Entrobacteriaceae* (MacConkey selective medium), *Lactobacillus* (MRS medium – agar), *Enteroccocus* (substrate – agar with kanamycin, esculin and sodium azide), and *Bifidobacterium* (Garche medium).

Analytical methods

During the digestion process, the total polyphenols content and the antioxidative potential of the digested materials were determined. The extraction process was carried out using a 70% solution of acetone employing a single extraction of polyphenols from the samples examined.

The digested material (3 mL) was mixed with 7 mL of acetone (≥99%) to obtain 70% concentration of acetone in the solution. The mixture was next shaken (shaker type KL-942) for one hour at a room temperature. Afterwards the samples were centrifuged at 3000 rpm and 300 μL of the supernatant was used for total polyphenols and antioxidative activity analysis.

The total polyphenols content was measured using a modified Folin-Ciocalteu method and its values were estimated from a standard curve of gallic acid. All results have been corrected for the presence of phenols in the pancreatin/bile salts mixture. Results are expressed as mg equivalents of gallic acid per g of digested products [Singleton & Rossi, 1965].

The antioxidative activity (TEAC) was determined against the ABTS reagent (2,2'-azinobis-(3-ethylbenzothiazoline-6--sulphonic acid) according to the method described by Re *et al*. [1999]. Results of the TEAC assay are expressed as the capability of antioxidants to scavenge ABTS radicals relative to that of Trolox (a water-soluble vitamin E analogue) and given as mg Trolox/g of the extract examined.

The number of viable bacterial cells was determined using the Koch's plate method. The number of microorganisms introduced into the digestion process amounted to about 10^6 cfu/mL.

Statistical analysis

Results obtained were subjected to the analysis of variance and a significance Tuckey test at $p \leq 0.05$. All analyses were determined in three replications.

RESULTS AND DISCUSSION

In order to identify the impact of individual plant extracts on the antioxidative properties, the concentration of phenolic compounds and the increase of intestinal microflora, food products (pasta, manufactured with and without the addition of plant extracts) were subjected to the process of "digestion". The addition of extracts to lentil flour resulted in the increase of both the concentration of phenolic compounds as well as of the antioxidative potential. However, the highest antioxidative potential was determined in the pasta manufactured with the addition of oregano extracts (Table 1, Figures 1 and 2).

The aqueous plant extracts obtained as well as the products manufactured with their addition were characterised by different initial concentrations of phenolic compounds and antioxidative activity. Pure extracts with the concentration of 50 mg/mL exhibited a low antioxidative potential but a relatively high content of phenolic compounds. The exception here were extracts obtained from oregano in which values of the parameters examined turned out to be the highest (respectively, 3.5 mg/g of polyphenols and 7.0 mg Trolox/g – antioxidative activity), (Table 1). There is little literature data concerning products with the addition of characteristic spices which, at the same time, lend the product high antioxidative potential. Research is still under way aimed at finding effective antioxidants among plant raw materials which would restrict significantly the above oxidation processes and, therefore, extend the stability of a developed product. So far, extracts

TABLE 1. Concentration of phenolic compounds and the antioxidative activity in the plant extract.

Extract	Total polyphenols content (mg gallic acid/g)	Antioxidant capacity (mg Trolox/g)
Garlic	1.21 ± 0.04^a	1.59 ± 0.26^a
Onion	1.73 ± 0.02^b	2.89 ± 0.19^b
Oregano	3.51 ± 0.11^c	7.02 ± 0.44^c

Values are means ± SD of three independent experiments. Means with different letters: $^{a, b, c, d}$ are significant (in the columns) at $p<0.05$.

of aloes, ginseng, rosemary, sage and tea catechin have been investigated to test their capabilities to control the oxidation processes. Out of the examined extracts, the most effective in protecting food products against lipid oxidation were: an extract from tea in the amount of 0.25%, rosemary – 0.10% and sage in the amount 0.05% [Mc Carthy *et al*., 2001; Tang *et al*., 2001].

Results obtained in this study indicate significant differences in the content of phenolic compounds as well as their antioxidative activity found at all stages of the performed process of digestion. The highest content of phenolic compounds was recorded in the case of all products after the process of digestion carried out in the "large intestine" – 3.21 mg/g – noodles with the oregano extracts (Figure 1). The highest antioxidative activity was determined at the stage of digestion in the "large intestine" for the products obtained from green lentil seeds, *i.e.* 32.43 mg Trolox/g – noodles with the oregano extract, whereas in pasta from lentil (without the addition of plant extracts) the antioxidative activity was recorded at a level of 22.12 mg Trolox/g (Figure 2).

Another factor which cannot be overlooked is the action of the intestinal microflora which, due to the metabolic processes taking place, primarily, in the large intestine, may increase the antioxidative potential of the digested products. In natural conditions, flavonoids occur, primarily, in the form of glycosides that might be hydrolysed by β-glycosides which can be either of bacterial origin [Walle *et al*., 2005]. During this process, phenolic compounds are probably liberated from the glycoside flavones as a result of hydrolysis of the glycosidic bond which occurs between the sugar residue and a hydro cyclic ring. The above transformation leads

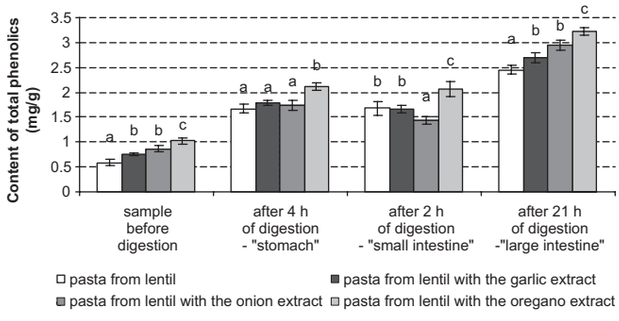

FIGURE 1. Changes in the concentration of phenolic compounds in products obtained from the green lentil seeds determined at the consecutive stages of the "digestion" process.

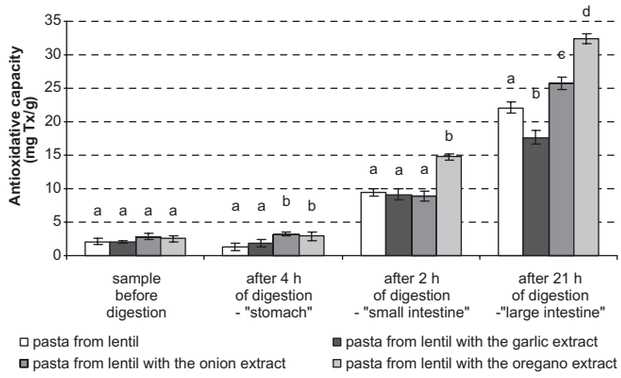

FIGURE 2. Changes in the antioxidative capacity in products obtained from the green lentil seeds determined at the consecutive stages of the "digestion" process.

to the development of an active aglycone which is more reactive than the glycoside form and which can be recognised as one of the more favourable changes taking place in the course of the *in vitro* digestion. Flavonoid metabolism produces a series of phenolic compounds that have been identified as aromatic acids, probably it may be the reason for the increase the antioxidative potential of the digested process too [Blaut *et al*., 2003]. In turn, seeds of leguminous plants constitute a rich source of proteins, oligosaccharides which act as excellent substrates for the growth and development of the intestinal microflora [Gawęcki & Libudzisz, 2006]. Assessing the quantitative changes of the intestinal microflora under conditions of the gastrointestinal tract *in vitro*, high survivability of all the four groups of bacteria was observed in the model conditions of the gastrointestinal tract *in vitro* during the process of digestion of the products obtained from green lentil without plant extracts. On the other hand, in the case of pastas with the addition of plant extracts, an inhibiting effect was observed in respect of the bacteria from *Enterococcus* and *Lactobacillus* genera. Following the digestion process at the level of "large intestine", their numbers were estimated at the level of 10^8 cfu/mL, whereas pastas without the inclusion of plant extracts stimulated the growth of the determined microorganisms whose numbers were estimated at the level of 10^9-10^{10} cfu/mL (Table 2). It is believed that traits that support the antimicrobial activity of extracts from plant compounds include hydrophobicity and their antioxidative capacity. Some flavonoids can inhibit proliferation and re-

TABLE 2. Change in the number of intestinal microflora in the course of digestion of selected products.

Microorganisms	Product (Log_{10} cfu/mL of digesta, mean ± SD)								
	Inoculum	Pasta from green lentil		Pasta from lentil with the garlic extract		Pasta from lentil with the onion extract		Pasta from lentil with the oregano extract	
		[1]2 h	[2]21 h	[1]2 h	[2]21 h	[1]2 h	[2]21 h	[1]2 h	[2]21 h
Bifidobacterium	6.49 ± 0.15	7.51 ±1.04	10.58 ±1.14	7.46 ±0.22	9.28 ±0.21	7.21 ±0.26	9.13 ±0.16	7.22 ±0.16	9.62 ±0.16
Lactobacillus	6.31 ± 0.19	6.7 5 ±1.11	9.79 ±1.09	7.52 ±0.1	8.19 ±0.12	7.14 ±0.18	8.83 ±0.12	7.19 ±0.16	8.39 ±0.16
Enterococcus	6.34 ± 0.24	7.11 ±1.09	9.59 ±0.45	7.60 ±1.30	8.33 ±0.25	7.16 ±0.37	8.34 ±0.35	7.31 ±0.16	8.23 ±0.16
Enterobacteriaceae	6.17 ± 0.11	7.22 ±0.89	9.31 ±1.06	7.32 ±0.27	10.47 ±0.36	7.22 ±0.16	9.40 ±0.20	7.15 ±0.16	9.17 ±0.16

[1]after the period of incubation in the "small intestine" together with the intestinal microflora; [2]after the period of incubation in the "large intestine".

duce the determined number of bacteria without damaging cells and thus enabling them to develop colonies. However, until now little is known about types of interactions constituting the basis of the phenomena taking place in the intestinal ecosystem [Vattem *et al*., 2004].

CONCLUSIONS

1. The products obtained with the addition of plant extracts exhibited a significant increase in polyphenol contents as well as their antioxidative activity before and during the digestion process in comparison to the products without the addition of plant extracts.

2. The highest antioxidative activity after the termination of the digestion process was determined in the case of noodles with the addition of the oregano extract (32.43 mg Trolox/g) at their relatively high total content of phenolic compounds (3.21 mg/g).

3. Products prepared from green lentil seeds enriched with plant extracts increased numbers of the assayed microorganisms, primarily, bacteria from the *Enterococcus* and *Lactobacillus* genera. After the digestion process at the stage of "large intestine", their counts were found at the level of 10^8 cfu/mL, whereas noodles without plant extracts failed to inhibit the development of these bacteria and their levels were determined at 10^9-10^{10} cfu/mL.

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Received June 2008. Revision received December 2008 and accepted January 2009.