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EFFECT OF PLANT EXTRACTS ADDITION ON PHENOLIC COMPOUNDS ACTIVITY AND INTESTINAL MICROFLORA INCREASE IN THE GASTROINTESTINAL TRACT MODEL

Małgorzata Gumienna, Małgorzata Lasik, Zbigniew Czarnecki

Institute of Food Technology of Plant Origin, Department of Fermentation and Biosynthesis, The August Cieszkowski Agricultural University of Poznań

Key words: intestinal microflora, polyphenols, antioxidative activity, legumes seeds

The aim of the presented research was to determine the activity and interactions of the human intestinal microflora with biologically-active compounds and to ascertain the degree of its "*in vitro*" survivability in the applied model of the gastrointestinal tract. Products (noodles) from colour bean seeds cv. Red Kidney were obtained after thermoplastic treatment with addition of plant extracts: garlic, onion and oregano (concentration of aqueous extracts 50 mg/mL). The highest antioxidant capacity (22.88 mg Tx/g) as well as content of phenolic compounds (2.94 mg/g) after the digestion process was determined for noodles from red bean with the onion extracts. The growth of investigated bacteria (*Enterobacteriaceae*, *Lactobacillus, Enterococcus, Bifidobacterium* genera) was affected by the digested noodles from red bean with the plant extracts, especially of the *Enterococcus and Bifidobacterium* species. The amount of bacteria after digestion in the large intestine maintained at 10^8 cfu/mL, whereas the noodles without the addition of plant origin extracts also stimulated the growth of microflora. In effect, the amount of bacteria detected in the large intestine accounted for $10^9 - 10^{10}$ cfu/mL.

INTRODUCTION

The human organism is a very complicated "machine" and its principles of action have always fascinated people, including scholars. The knowledge of processes associated with the digestion of food products by humans provides a lot of valuable information about the assimilation and bioavailability of nutrients. If the "*in vitro*" process simulates the course of digestion faithfully, then even experiments conducted under "*in vivo*" conditions do not give such an accurate picture. Therefore, "*in vitro*" systems provide a good alternative for investigations carried out on humans and animals [Aura *et al*., 1999; Gil-Izquierdo *et al*., 2001]. Colonization of the gastrointestinal tract by the appropriate intestinal microflora is also very important. It constitutes the most important and complex ecosystem made up of approximately 500 different species of microorganisms. These microorganisms colonize the area of nearly 400 m^2 of the intestinal epithelium and make up the total of about 10^{14} cells [Gawęcki & Libudzisz, 2006].

Legumes have been cultivated by man since times 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. They act as compounds inactivating free radicals, form complexes with metals, inhibit activities of lipoxygenases as well as other enzymes catalysing oxidation reactions [Mc Carthy *et al*., 2001]. Therefore, the addition of substances which exert an antioxidising influence may extend the stability of food products by protecting them against the appearance of undesirable sensory traits, harmful constituents and loss of vitamins. Many antioxidants – contained, primarily, in spices and herbs – such as: oregano, thyme, marjoram or rosemary, are little applicable because of their very specific aroma which they introduce into food articles. 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, there is still very little data concerning the behavior of these compounds in the man's gastrointestinal tracts, in particular, 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 and to ascertain the degree of its "*in vitro*" survivability in the applied model of the gastrointestinal tract.

MATERIALS AND METHODS

The experimental material comprising seeds of colour bean cv. Red Kidney was obtained from the 2006 harvest. The following materials were used in experiments carried out in the developed model of the "*in vitro*" gastrointestinal tract: extracts of plant origin: garlic, onion (commercially available) and oregano("Kamis" company), products (noodles) from

Author's address for correspondence: Małgorzata Gumienna, Institute of Food Technology of Plant Origin, Department of Fermentation and Biosynthesis, The August Cieszkowski Agricultural University of Poznań, Wojska Polskiego 31, 60-624 Poznań, Poland; tel.: (4861) 8 4 87 267; faks: (4861) 84 87 314; e-mail: gumienna@au.poznan.pl

colour bean seeds after thermoplastic treatment with and without the addition of plant-originated 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 0.45 μm Millipore filters. The parameters of the thermoplastic treatment were selected on the basis of our own investigations and the experiments were carried out following methodology by Czarnecka *et al*. [1998]. The noodle products were obtained from the seed meal and wheat flour – semolina mixed at the ratio of 1:1.

Conditions of the "*in vitro*" digestion process. The "*in vitro*" digestion was conducted in a glass bioreactor equipped with 4 inlets allowing the introduction of the pH electrode, programming of the active acidity, supplemented of biochemical agents and appropriate media as well as collecting the 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 bioreactor was thermostable and the reactions were carried out at the temperature of 37°C. 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 but also taking into consideration studies carried out by Aura *et al*. [1999]; Gil-Izquierdo *et al*. [2001] and Knarreborg *et al*. [2002].

The employed intestinal microflora was collected from faeces of 3 volunteers 25 to 30 years of age. The standardisation of the intestinal microflora was conducted in accordance with the methodology prepared by Knarreborg *et al*. [2002]. The intestinal microflora was introduced into the system at the stage of digestion imitating conditions found in the small intestine (pH 7.4 maintained with the assistance of 1 mol/L $NaHCO_3$). The digestion process was carried out for 2 h at the temperature of 37°C and then the pH was increased to the level of 8.0 and the process was continued for another 18 h so as to imitate conditions occurring in the large intestine. Anaerobic conditions were maintained with the aid of the technical gas (nitrogen) which was passed through the bioreactor.

Sample collection. Samples for analyses were collected before "digestion" (initial raw material), following the stage of "stomach" digestion as well as after the passage of the experimental material through the "small intestine" and "large intestine". Simultaneously, at individual stages of "digestion", inoculations were made onto selective media assessing numbers of selected microorganisms. The determined groups of microorganisms included: *Enterobacteriaceae* (MacConkey selective medium), *Lactobacillus* (MRS medium – agar), *Enterococcus* (substrate – agar with kanamycin, esculin and sodium azide), *Bifidobacterium* (Garch's medium). Inoculated media were incubated in relatively anaerobic or anaerobic conditions depending on the determined group of microorganisms for the period of 72 h at temperatures ranging from 37°C to 42°C. The number of live 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.

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

The total polyphenolics content in the extracts was determined employing the reaction with the Folin and Ciocalteu phenolic reagent according to the method developed by Singleton & Rossi [1965]. The relative abilities of antioxidants to scavenge radical cation $ABTS^+$ were measurement by means of a spectrophotometric technique in comparison with the antioxidant potentioal of Trolox according to the method described by Re *et al*. [1999].

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

RESULTS AND DISCUSSION

The obtained aqueous plant extracts 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 mL/1 mL exhibited a low antioxidative potential but a relatively high content of phenolic compounds. The exception here were the extracts obtained from oregano in which the highest values of the examined parameters were recorded (respectively, 3.5 mg/g of polyphenols and 7.0 mg Tx/g – antioxidant activity) (Figure 1).

In order to check the impact of individual plant extracts on antioxidative properties, the concentration of phenolic compounds and the increase of intestinal microflora, the examined food products (noodles manufactured with and without the addition of plant additives) were subjected to the process of "digestion". The addition of extracts to bean 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 noodles manufactured with the addition of onion extracts (Figure 2 and 3).

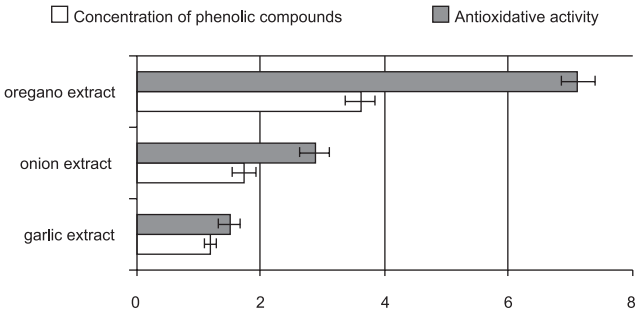

FIGURE 1. Concentration of phenolic compounds (mg/g) and the antioxidative activity (mgTx/g) in the plant extract.

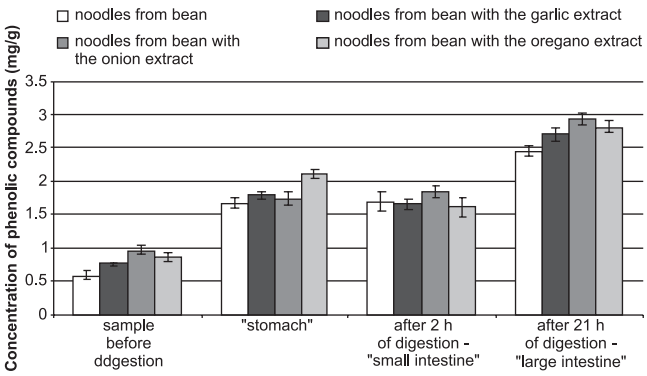

FIGURE 2. Changes in the concentration of phenolic compounds in products obtained from the colour bean seeds of cv. Red Kidney determined at the consecutive stages of the "digestion" process.

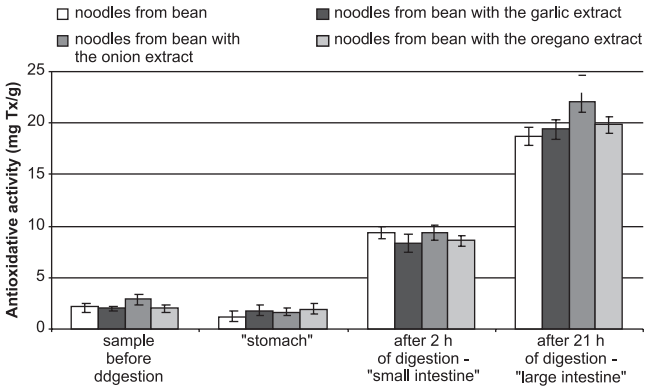

FIGURE 3. Changes in the antioxidative activity in products obtained from the colour bean seeds of cv. Red Kidney determined at the consecutive stages of the "digestion" process.

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 aiming 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 of aloes, ginseng, rosemary, sage and tea catechin have been investigated to test their capabilities to control oxidation processes. Out of the examined extracts, the most effective in protecting food products against lipid oxidation were: extract from tea at a concentration of 0.25%, rosemary – 0.10% and sage at a concentration of 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 "stomach" (2.20 mg/g – noodles with the oregano extract and *ca*. 1.7 mg/g – other products) and the "large intestine" (2.94 mg/g – noodles with the onion extract, 2.75 mg/g – noodles with the garlic and oregano extracts, 2.45 mg/g – without the plant extracts) (Figure 2). However, the highest antioxidative capacity was determined at the stage of digestion in the "large intestine" in which for the products obtained from colour bean seeds, it amounted to: 19.42 mg Tx/g – noodles with the garlic extract, 22.88 mg Tx/g – noodles with the onion extract and 19.41 mg Tx/g – noodles with the oregano extract, whereas in noodles from bean (without the addition of plant extracts) the antioxidative activity was found to account for 18.12 mg Tx/g (Figure 3).

In natural conditions, flavonoids occur, primarily, in the form of glycosides which can be hydrolysed within several minutes already in the human mouth by β-glycosides which can be either of bacterial origin or can originate from the exfoliating epithelial cells. This, however, depends on the food matrix and the structure of flavonoids themselves [Walle *et al*., 2005]. Therefore, the determined relatively high content of antioxidants at the stage of stomach digestion can be attributed to the activity of digestive enzymes. During this process, phenolic compounds are probably liberated from the glycoside flavones as a result of hydrolysis of the glycoside bond which occurs between the sugar residue and hydro cyclic ring. The above transformation leads to the development of an active aglycone which is more reactive than the glycoside form and which can be recognized as one of the more favorable changes taking place in the course of the "*in vitro*" digestion [Gao *et al*., 2006]. 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, can increase the antioxidative potential of the digested products [McCue & Shetty 2005]. Therefore, in order to assess the influence of plant extracts on the intestinal microflora as well as its interaction with polyphenols during the process of digestion, intestinal microflora (pH – 7.4, small intestine) isolated from faeces of a mature person was introduced into the experimental model. Assessing the quan-

TABLE 1. Quantitative changes of the intestinal microflora in the course of digestion of selected products.

Microorganisms	Product (Log_{10} cfu/mL of digesta, mean±SD)								
	Inoculum	Noodles from bean		Noodles from bean with garlic extract		Noodles from bean with onion extract		Noodles from bean with oregano extract	
		[1]2h	[2]21h	[1]2h	[2]21h	[1]2h	[2]21h	[1]2h	[2]21h
Bifidobacterium	6.49±0.15	7.52±1.04	10.68±1.14	7.46±0.22	8.28±0.21	7.20±0.26	8.13±0.16	7.22±0.16	8.52±0.16
Lactobacillus	6.31±0.19	6.71±1.11	9.79±1.11	7.51±0.1	8.69±0.12	7.14±0.18	8.18±0.12	7.19±0.16	9.49±0.16
Enterococcus	6.34±0.24	7.12±1.09	9.56±0.45	7.40±1.30	8.33±0.25	7.16±0.37	8.34±0.35	7.31±0.16	8.83±0.16
Enterobacteriaceae	6.17±0.11	7.20±0.89	9.37±1.06	7.32±0.27	10.27±0.36	7.22±0.16	9.40±0.20	6.14±0.16	9.13±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"

titative changes of the intestinal microflora in 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 colour beans without plant extracts. On the other hand, in the case of pastas in which garlic and onion extracts were applied, an inhibiting effect in relation to the bacteria from *Enterococcus* and *Bifidobacterium* genera was observed. 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 1). It is believed that traits that support the antimicrobial activity of extracts from plant compounds include hydrophobicity and their antioxidising activity. Some flavonoids can inhibit proliferation and reduce the determined number of bacteria without damaging cells and making allowing them to develop colonies [Vattem *et al*., 2004]. Investigations carried out so far showed that the assimilabilty of the majority of antioxidants present in food products depends significantly on the activity of the present intestinal microflora. However, until now little is known about what kind of interactions constitute the basis of the phenomena taking place in the intestinal ecosystem [Gao *et al*., 2006].

CONCLUSIONS

1. The obtained products with the addition of plant extracts exhibited a significant increase of polyphenol contents as well as their antioxidative activity before and during the digestion process.

2. The highest antioxidant activity after the termination of the digestion process was determined in the case of noodles with the addition of the onion extract (22.88 mg Tx/g) at their relatively high total content of phenolic compounds (2.91 mg/g).

3. Products prepared from colour beans seeds supplemented with plant extracts increased numbers of the determined microorganisms, primarily, bacteria from the *Enterococcus* and *Bifidobacterium* genera. After the digestion process at the stage of "large intestine", their numbers were found at the level of 10^8 cfu/mL, whereas noodles in which no plant extracts were added failed to inhibit the development of these bacteria and their levels were determined at 10^9-10^{10}cfu/mL.

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WPŁYW DODATKU EKSTRAKTÓW ROŚLINNYCH NA AKTYWNOŚĆ ZWIĄZKÓW FENOLOWYCH I WZROST MIKROFLORY JELITOWEJ W MODELU PRZEWODU POKARMOWEGO

Małgorzata Gumienna, Małgorzata Lasik, Zbigniew Czarnecki

Instytut Technologii Żywności Pochodzenia Roślinnego, Zakład Fermentacji i Biosyntezy, Akademia Rolnicza im. Augusta Cieszkowskiego, Poznań

W pracy oceniano zmiany mikroflory jelitowej i jej interakcje ze związkami aktywnymi biologicznie. Do badań wybrano makarony otrzymane z nasion fasoli kolorowej odmiany Red Kidney, z dodatkiem ekstraktów roślinnych uzyskanych z czosnku, cebuli i oregano (koncentracja wodnych ekstraktów 50 mg/mL). Największą aktywność antyoksydacyjną (22,88 mgTx/g) oraz całkowitą zawartość polifenoli (2,94 mg/g) po zakończonym procesie trawienia oznaczono w makaronach otrzymanych z fasoli kolorowej z dodatkiem ekstraktów z cebuli. Ponadto w wyniku przeprowadzonych badań stwierdzono, że poddane procesowi „trawienia" makarony z fasoli kolorowej z dodatkiem ekstraktów roślinnych wypływały na wzrost oznaczanych grup mikroorganizmów (*Enterobacteriaceae*, *Lactobacillus, Enterococcus, Bifidobacterium)*, głównie bakterii z rodzaju *Enterococcus* i *Bifidobacterium*. Po przeprowadzonym procesie trawienia na etapie „jelita grubego" odnotowano ich liczbę na poziomie 10^8 j.t.k./mL, podczas gdy makaron bez dodatku ekstraktów stymulował wzrost oznaczanych drobnoustrojów i ich liczbę oznaczono na poziomie 10^{9} – 10^{10} j.t.k./mL.