

Stimulating and protective effects of peptides from chicken embryo extract on probiotic bacteria

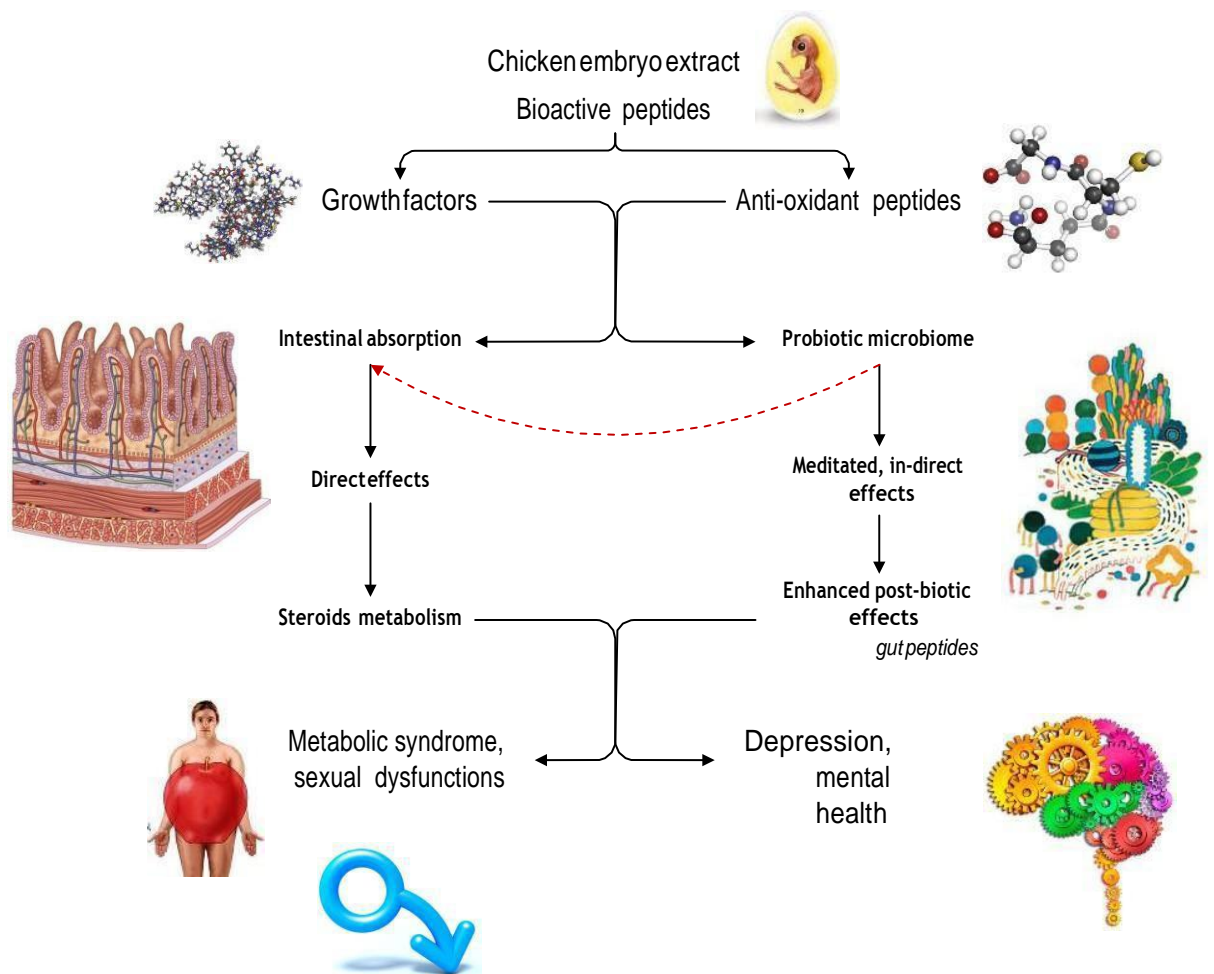
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Abstract

We evaluated the influence of peptides from chicken embryo extracts on probiotic bacteria. We separated from Humanofort, an extract from chicken embryos standardized in embryo peptides, two peptides fractions, one with a more significant anti-oxidant activity (F1) and other one which exert also a growth stimulation effect on probiotic bacteria (F2). The anti-oxidant peptides fraction protect probiotic bacterial strains from two genera, *Lactobacillus* and *Bifidobacterium*, against (nitro)oxidative stress induced by (micro)aerobic conditions. The peptides fraction which include growth factors stimulate the development of probiotic bacteria, especially under anaerobic conditions. We proposed two different physiological mechanisms involved into beneficial effects on human health of orally ingested chicken embryo / partially incubated hen eggs: a direct one, resulted from the intestinal absorption of peptides with anti-oxidant and growth factors activity, and an in-direct, mediated one, due to an enhanced post-biotic effect of probiotic microbiome.



Graphical abstract

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Extracts from chicken embryos / partially incubated fertilized hens eggs have been shown to improve health status of human subjects. Humanofort® (Hipocrate 2002 Srl, Bucharest, Romania), an extract from chicken embryos, standardized in embryo-peptides, consumed daily at a dose of 4 caps x 50 mg, induced a significant modification on lipid metabolism in third age human subjects. On treated subjects total cholesterol and LDL cholesterol decreased by 30% as compared to initial values (3). In 80% of the patients increase of HDL cholesterol and decrease of insulin levels in blood plasma were also observed. After 60 days administration of Humanofort extract determined a significant reduction of cortisol, associated with modifications of Protein C Reactive (CRP) and Insulin-like Growth Factor (IGF-1) (4). A preparation from partially incubated hen eggs was proposed for the prophylactic and/or therapeutic treatment of different memory disorders, in human subjects (5).

Traditionally, embryonated avian eggs are considered healthy food products in south-east Asia. One of the most famous Chinese Pharmacopeia, Compendium of Materia Medica, includes chicken embryonated eggs among the contributors to the immune function enhancement (6). Balut, boiled embryonated duckling eggs, is considered an aphrodisiac food in Philippines (7). Experimental approaches confirmed this ethnopharmacological knowledge. Li *et al* (6) find that chicken embryo eggs extract is a potent immune stimulator on the immunosuppressed mouse. Eskeland *et al.* (8) confirmed, in a two double-blind cross-over placebo-controlled experiments, the significant enhancing effect on sexual desire, in men with normal and reduced sexual drive, of the extract from partially incubated hen eggs.

There were described several mechanisms involved into this beneficial effects on human health of orally ingested chicken embryo / partially incubated hen eggs. On the patent application related to Rellidep (9) the anti-depressant effect of Isolate A, which contain a large amount of a 30173 Da peptide, is claimed to be mediated by or associated with one of more glutamate receptors or by neurokinin 2 (NK2) receptors. The anti-stress effect of YTE, a preparation which contain oligo-peptides extracted from partially incubated hen eggs, was claimed to be related to normalization of the cortisol values (10, 11). Similar effects on normalization of steroidogenesis was found also for Humanofort, the chicken embryo extract standardized in embryo-peptides (12). Immunostimulating effects of embryonated eggs digested extracts were associated with a significantly enhanced spleen lymphocyte proliferation and interleukin 2

secretion (for lipophilic extracts), and elevated peritoneal macrophage phagocytosis and nitric oxide (NO) production activity (for water soluble extracts) (5). Humanofort active ingredients include peptides with cyto-stimulating and cyto-protective effects (13). Peptides with anti-oxidant effects were identified and purified from incubated fertilized hen eggs (14).

The extracts from chicken embryos / partially incubated fertilized hens eggs are orally administrated, as healthy food / nutraceuticals. Beneficial effects are thus driven by the intestinal absorption of the active ingredients, considered to be different categories of (embryo) peptides. Research in humans has shown that peptides of different sizes can pass through the intestinal epithelium (15), but their absorption and further expression of their biological activity, is mediated by the interaction with the gut epithelium and microbiota (16). Microbiota could be considered as part of intestinal barrier because is one important modulatory factor involved in its regulation (17). Till now were not done investigation regarding the effect of chicken embryo / embryonated eggs peptides on the development of probiotic bacteria, thus the bifidogenic activity of peptides from human milk (18) and from lactoferrin (19) was demonstrated. Besides that, an effect on development of probiotic microbiome could be also involved, through an enhanced post-biotic effect, on the physiological mechanisms related to the beneficial effects on human health of orally ingested chicken embryo / partially incubated hen eggs.

We investigated in this study the stimulating and the protective effects on several probiotic bacteria of our standardized embryo extract (Humanofort) and its peptides components.

Materials and methods

Tested bioactive preparation and peptides. Humanofort is a standardized extract of chicken embryo, obtained according to the following process (12): chicken embryos harvesting from partially incubated hen eggs and their disintegration, dilution with sterile water, homogenization and dissociation of embryonic growth factors from their soluble receptors; embryo-peptides concentration through tangential ultrafiltration; embryo-protein denaturation and mixing of embryo-peptides with denatured proteins; obtainment of cationic peptides and their addition to the mixture embryo peptides – embryo proteins, homogenization and spray-drying of the final mixture.

From this extract we separated two peptides components by chromatographic gel filtration. The resulted Humanofort powder was re-suspended in double distillate water, centrifuged at 10,000 g for 20 min (to avoid any further gel-chromatographic bed warping) and gel-chromatographed on a preparative columns (Sephadex G-50). The compounds from fractions included in chromatographic gel (with molecular weight under 50 kDa) was unified and freeze-dried. The freeze-dried powder was reconstituted in ultrapure water (obtained on a Milli-Q Integral Water Purification

System, EMD Millipore Billerica, MA, USA), centrifuged for 15 min at 15,000 g and high performance gel chromatographed, on a DX 500 system (Dionex Thermo Scientific, Sunnyvale, CA, USA) using a TSKgel® G2000SW_{XL} (Tosho Corporation, Tokyo, Japan) column. The column was previously calibrated with bovine serum albumin (67kDa), ovalbumine (42 kDa), hemoglobin (18 kDa) and cytochrome c (12.7 kDa). The gel-filtration was done during 30 min, with ultrapure water as mobile phase, at a flow of 0.5 ml per min and a pressure of 2.7 MPa. The separation process was UV monitored, at two different wave length (220 nm and 280 nm). Two fractions was separated: one with a molecular weight of 10 KDa (F1) and the other one with a molecular weight of 5 KDa (F2). The peptide concentration in F1 and F2 was measured with a modified Lowry method, using a Sigma kit (P5656).

Bacterial strains and culture conditions. We used probiotic bacterial strain from *Lactobacillus* and *Bifidobacterium* genera. *Lactobacillus helveticus* strain R0052 (Rosell 52) and *Lactobacillus rhamnosus* strain R0011 (Rosell 11) were provided by Institut Rosell - Lallemand Inc. (Montreal, QC, Canada). Type strains of *Bifidobacterium*, *B. adolescentis* DSM 20083^T, *B. breve* DSM 20213^T and *B. longum* DSM 20219^T, were purchased from the Deutsche Sammlung für Zellkulturen und Mikroorganismen (Braunschweig, Germany).

We prepared stock cultures by mixing a fresh culture with sterile 20% glycerol and sterile 20% (w/v) reconstituted skim milk, in a 2:5:5 ratio, with subsequent storage at - 80°C, in portion of 1 ml placed in 2 ml vials (Greiner Bio-one, Frickenhausen, Germany). Before each independent assay, we prepared fresh cultures by adding one thawed stock culture to 9 ml of Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany), enriched with ascorbic acid (0.1%). We incubated the resulted probiotic bacteria cultures between 12 and 16 h at 37°C, in an anaerobic jar containing Anaerogen sachets (Oxoid Thermo Scientific, Hampshire, UK). The incubation period was variable, each bacterial culture being collected once it had reached the beginning of the stationary growth phase. Concentrations of bacteria suspensions were quantified through optical density (OD) measurement at 630 nm in order to standardize the inoculum at $7.0 \pm 0.5 \log_{10}$ cfu/ml.

Determination of ABTS radical cation scavenging activity. We performed this assay of the antioxidant activity of Humanofort and its embryopeptides components, according to Re *et al.* (20). ABTS radical cation, generated by incubation of ABTS (7 mM) with potassium persulfate (2.45 mM) for 12–16 h in dark, was further diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. In order to evaluate the scavenging activity, tested products were mixed with ABTS radical cation solution in a total volume of 2 ml. The decrease in absorbance at 734 nm was measured after 6 min of reaction at 30 °C. Glutathione was used as positive control. % ABTS radical cation scavenging activity was calculated using the following formula: $100 \times (A_c - A_s/A_c)$, where A_c is the

absorbance of the control and A_s is the absorbance of the tested sample, Humanofort, its peptide components or glutathione.

Determination of superoxide anion radical scavenging assay. For this assay of anti-oxidant activity of Humanofort and its peptides component we used the method of Marklund and Marklund modified by Wang (21), with some minor adaptations to the conditions of our lab. The reaction mixture (3.1 ml) contained different concentrations of extracts (50–200 $\mu\text{g/ml}$), 1 mM EDTA in 50 mM Tris-HCl buffer at pH 8.0 and 6 mM pyrogallol. The increase in absorbance at 325 nm was measured every 30 s for 4 min. Glutathione was the positive control. % superoxide anion radical scavenging activity was calculated by the formula: $100 \times (S_c - S_p/S_c)$ where S_c and S_p are the slopes of the plots of absorbance vs. time for control and samples, respectively.

Determination of hydroxyl radical scavenging activity. We determined the antioxidant activity on this assay according to the method reported by Jeong *et al.* (22). The reaction mixture, containing tested products, 1.5 mM iron (II) sulfate heptahydrate, 20 mM sodium salicylate and 6 mM hydrogen peroxide in a total volume of 2.4 ml, was incubated at 37°C for 30 min. After cooling, the absorbance was measured at 562 nm. L-Ascorbic acid was used as positive control. % hydroxyl radical scavenging activity was calculated using the formula: $100 \times (A_c - A_s/A_c)$, where A_c is the absorbance of the control and A_s is the absorbance of the tested sample, Humanofort, its peptide components or ascorbic acid.

Determination of lipid peroxidation inhibition activity. We determine the inhibition of lipid peroxidation by Humanofort and its peptides component by using the method described by Choi *et al.* (23) with minor changes. In brief, the reaction mixture, containing the tested samples, 20 mM linoleic acid, 4 mM iron (II) sulfate heptahydrate, 2 mM ascorbic acid and 100 mM Tris buffer (pH 7.5) in a total volume of 1.3 ml, was incubated at 37°C for 30 min. A volume of 1 ml of 5.5% trichloroacetic acid and 1% thiobarbituric acid was further added followed by heating at 95°C for 20 min. After cooling and centrifugation, the absorbance was measured at 532 nm. We used trolox, (\pm)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Sigma-Aldrich, St. Louis, MO, USA) as a positive control. % lipid peroxidation inhibition was calculated using the following formula: $100 \times (A_c - A_s)/(A_c - A_b)$ where A_c is the absorbance of the control, A_s is the absorbance of the tested sample, Humanofort, its peptide components or trolox, and A_b is the absorbance of the mixture containing only linoleic acid and Tris buffer.

Determination of NO scavenging activity. We determined the antioxidant activity of Humanofort and its peptides components also by determining the NO-scavenging activity according to the method of Sreejayan and Rao (24), with small modifications. Briefly, 60 μl of serial diluted sample extract was pipetted into a 96-well flat-bottomed plate. Following this, 60 μl of 10 mM sodium nitroprusside dissolved in PBS was added into each well and the plate was then incubated under light at room temperature for

150 min. Finally, an equal volume of commercial Griess reagent (Sigma Aldrich), containing 0.2% naphthylethylenediamine dihydrochloride and 2% sulphanilamide in 5% phosphoric acid was added into each well in order to measure the nitrite content. After 30 min of incubation necessary for the formation of diazonium salts, we measured the absorbance at 540 nm. L-Ascorbic acid was the positive control. % nitric oxide scavenging activity was calculated as $100 \times (A_c - A_s/A_c)$, where A_c is the absorbance of the control and A_s is the absorbance of the tested sample, Humanofort, its peptide components or ascorbic acid.

Effect of MRS supplementation with Humanofort or its peptide components on growth of probiotic bacteria. We obtained the growth curves for each strain used in this study by reading optical density (OD) using a microplate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland) at 630 nm after the incubation period. Growth behavior of the strains, under both aerobic and anaerobic conditions was followed on different media: a) un-supplemented lactobacilli MRS medium, b) MRS supplemented with different concentrations of Humanofort (100 μ g/ml) or its peptide components (F1, 90 μ g/ml, F2, 10 μ g/ml) to study strain growth in medium containing embryo-peptides and c) MRS supplemented with ascorbic acid (0.1%) as a positive control medium and to study growth of strains in a medium with reduced oxygen level. Ascorbic acid was used for comparison because it is a powerful oxygen scavenger and its addition often improves the growth patterns of anaerobic cultures in aerobiosis (25). Concentration range of Humanofort and its peptide components was selected on the basis estimated concentrations locally resulted in the intestinal lumen after the ingestion of 1 g (2 x 500 mg Humanofort powder included in enteric capsules).

Two wells of the microplate were filled with 200 μ l of each medium to obtain a duplicate reading for each strain and inoculated with 20 μ l of fresh cultures. To further reduce the presence of oxygen in the medium, 50 μ l sterile mineral oil were added to wells on the surface of the inoculated media (we will further referred to this as the “anaerobic conditions”). The plates were incubated for 24 h at 37°C. OD measurements (630 nm) were taken every 15 min, with the microplate reader agitation set at “moderate shaking” for 30 s before each OD reading. Three independent assays were carried out in this experiment. Maximal OD (OD_{max}) obtained were determined. To compare the growth rate of the bacteria tested, OD data were transformed into ln values of biomass and plotted against time. The slope of the exponential growth part of the curve, giving the maximum growth rate (μ_{max}), was obtained by a simple linear regression of the ln scale plot (Sigma Plot Software, Jandel, Chicago, USA). Statistical analyses were performed using SigmaPlot software (Systat Software, Inc., San Jose, USA) on results obtained for the growth of bacteria. A one-way analysis of variance (ANOVA) at a significance level of 0.05 was performed on OD_{max} and μ_{max} results.

Effects of addition of Humanofort, its peptide components and ascorbic acid on the redox potential. To evaluate the impact of embryo-peptides and ascorbic acid

addition on the medium, the ORP values of lactobacilli MRS culture media with added concentrations of Humanofort or its peptides components or with 1000 µg/ml ascorbic acid were measured using a VWR Symphony platinum electrode (VWR Scientific, West Chester, USA) filled with a solution of Ag/AgCl. This electrode was connected to a VWR Symphony portable SP20 pH/ISE meter. The electrode reading was verified with a homemade solution of potassium ferrocyanide and potassium ferricyanide having an ORP value of + 234 mV (26). Three independent replicates were carried out for each condition and the data presented are the average of these three assays.

Fourier transform infrared spectroscopy analysis. In order to evaluate the effect of the presence or absence of oxygen or the presence of anti-oxidant (Humanofort, its peptide components, ascorbic acid) in the culture medium on the lipid molecular structure in the phospholipid bilayer of bacterial membrane, we made a Fourier transform infrared (FTIR) spectroscopy analysis. A 10% inoculum of fresh cultures of *L. helveticus* R0052 was grown at 37°C in 50 ml test tubes in control MRS broth in the presence or absence of oxygen or in MRS supplemented with 100 µg/ml Humanofort in the presence of oxygen. A moderate agitation of the test tubes was carried out every 2 h for fermentations in the presence of oxygen, while test tubes were incubated in anaerobic jars containing Anaerogen sachet (Oxoid) for those in the absence of oxygen. Fermentations were stopped when the cultures had reached the beginning of the stationary growth phase. Cells were pelleted by centrifugation at 9800 rpm for 5 min at 4°C and washed twice with deionized water. The bacterial paste obtained was placed on a CaF₂ window and stove-dried 15 min at 50°C to form a transparent bacterial film(27). Infrared spectra were measured at room temperature with a FT-IR Tensor 27 – Bruker spectrometer (Bruker Optik GmbH, Karlsruhe, Germany). FTIR spectra were recorded from 4000 to 900 cm⁻¹ at a resolution of 2 cm⁻¹. Each spectrum represents an average of 128 scans and is apodized with a Happ-Genzel function. The sample chamber of the spectrometer was continuously purged with dried and CO₂-free air to prevent atmospheric water vapor obscuring the bands of interest. Water vapor subtraction was undertaken when necessary. Spectral processing was performed using Omnic software (version 3.1, Thermo Electron Corporation). The scale of each spectrum was normalized to minimize differences due to sample amount and the baseline was corrected in the spectral region of 3000–2800cm⁻¹.

Results and discussions

We evaluated the free radical scavenging activity of Humanofort and its peptides components against the synthetic nitrogen-centered 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation, according to Re *et al.* (19) method. At the highest concentration tested (100 µg/ml), tested products scavenged 88.57 ± 0.63 (Humanofort) and, respectively, 98.42% ± 0.18% and 82.65% ± 0.62% of the radical (F1 and F2 peptides). Glutathione, the positive control, completely scavenged the

radical (Figure 1a). According to the EC₅₀ values F1 peptides ($21.29 \pm 0.17 \mu\text{g/ml}$) was the most active; glutathione scavenged the radical with an EC₅₀ value of $3.42 \pm 0.05 \mu\text{g/ml}$, in our experimental conditions (Table 1). Glutathione is a powerful antioxidant peptides, thus is approximately six times less potent, F1 peptides is an efficient ABTS radical scavenger (considering also its molecular mass of 10 kDa, approx. 30 times higher than that of glutathione).

Further, we evaluated the scavenging activity of Humanofort and its peptide components against superoxide, hydroxyl and nitric oxide radicals, according to methods already presented [20–22]. Superoxide anion radical, generated by the autoxidation of pyrogallol, was efficiently scavenged by Humanofort and F1 peptide. The superoxide scavenging activity of F1 increased dose-dependently from $8.21\% \pm 0.56\%$ at $10 \mu\text{g/ml}$ to $96.15\% \pm 0.43\%$ at $50 \mu\text{g/ml}$. In the same concentration range ($10\text{--}50 \mu\text{g/ml}$), the scavenging effects of glutathione varied between $92.12\% \pm 0.61\%$ and $100.91\% \pm 0.71\%$ (Figure 1b).

Hydroxyl radical, generated by Fenton reaction was scavenged with different potencies by Humanofort and its peptides components. At 1 mg/ml , the scavenging percentages of Humanofort and its F1 and F2 peptide components were $49.24\% \pm 0.78\%$, $79.05\% \pm 0.27\%$ and $40.56\% \pm 0.28\%$, respectively. The positive control, L-ascorbic acid, scavenged hydroxyl radical more efficiently than Humanofort and its peptides components; it completely scavenged the radical at a concentration range of 1 mg/mL (Figure 1c).

Nitric oxide was generated by the decomposition of sodium nitroprusside and measured on the basis of Griess reaction [23]. The nitric oxide scavenging activity of Humanofort and its F1 and F2 peptides increased from $8.40\% \pm 0.59\%$, 9.72 ± 0.62 and $2.35\% \pm 0.08\%$, respectively (at $10 \mu\text{g/ml}$) to $88.71\% \pm 0.34\%$, 94.23 ± 0.84 and $63.56\% \pm 0.83\%$, respectively (at $100 \mu\text{g/mL}$). Within the same concentration range, the scavenging activity of L-ascorbic acid increased from $38.01\% \pm 0.46\%$ to $71.62\% \pm 0.40\%$. We could note the excellent nitric oxide scavenging capacity of Humanofort and its F1 peptide component, in our experimental conditions, in comparison to that of the positive control, L-ascorbic acid (EC₅₀ = $42.23 \pm 0.41 \mu\text{g/ml}$) (Table 1).

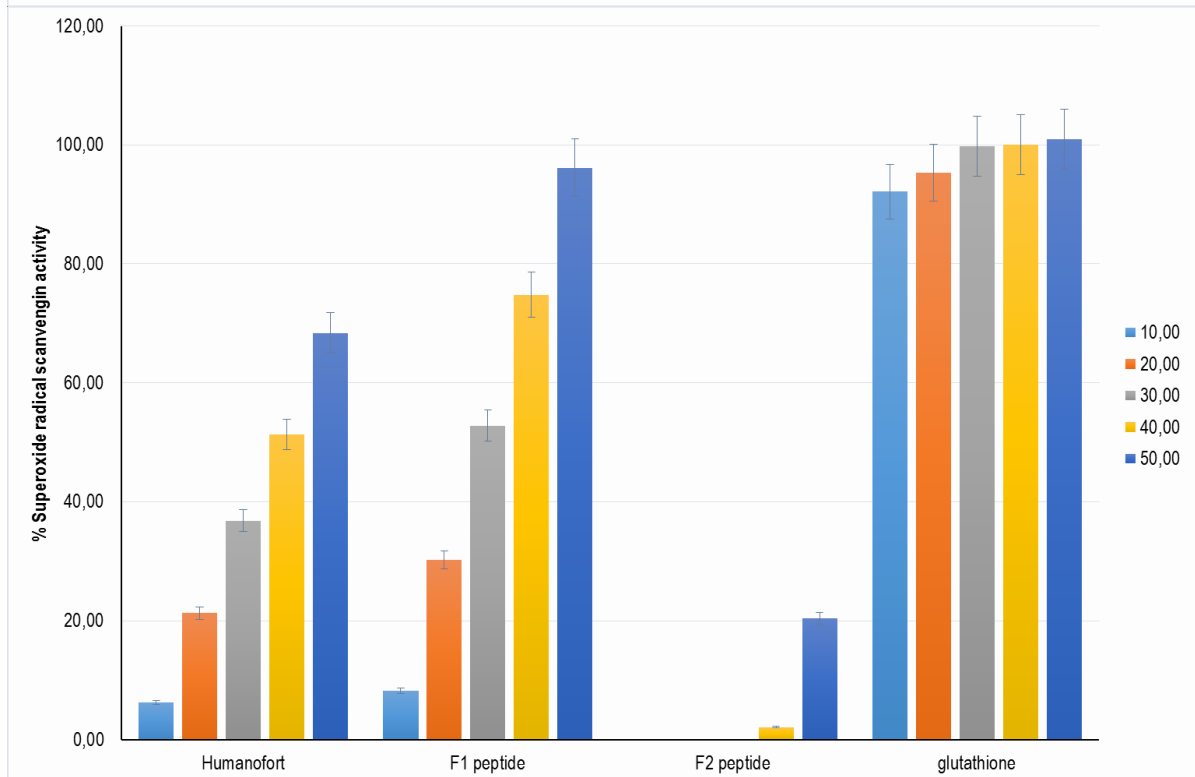
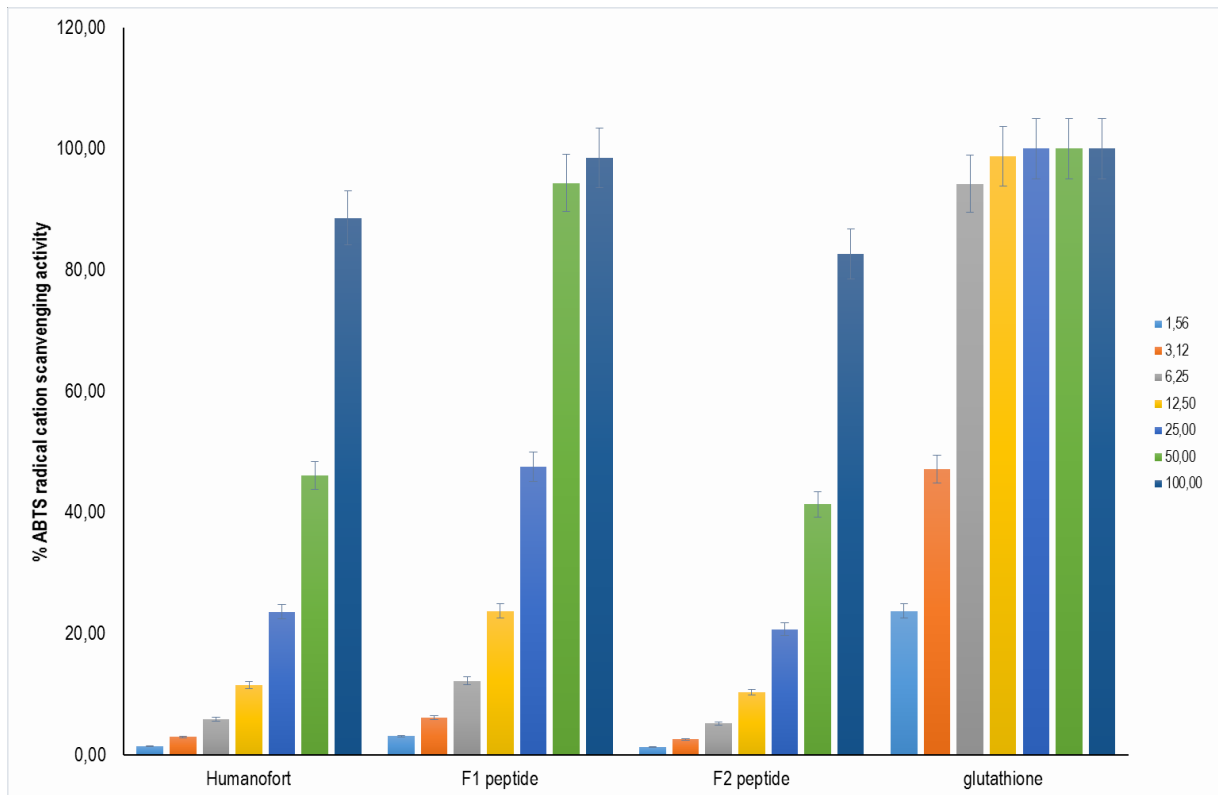
We tested also Humanofort and its peptides components effects on a lipid peroxidation inhibition assay. In this assay Humanofort and its peptides components, at 1 mg/ml , have a lipid peroxidation inhibitory effects of $58.61\% \pm 0.61\%$ (Humanofort) $74.52\% \pm 0.96\%$ (F1) and $40.73\% \pm 0.48\%$ (Figure 1e). The EC₅₀ values revealed that F1 peptide possessed the highest lipid peroxidation inhibitory activity (EC₅₀ = $210 \pm 0.07 \mu\text{g/ml}$). According to the EC₅₀ values, the lipid peroxidation inhibitory effect of

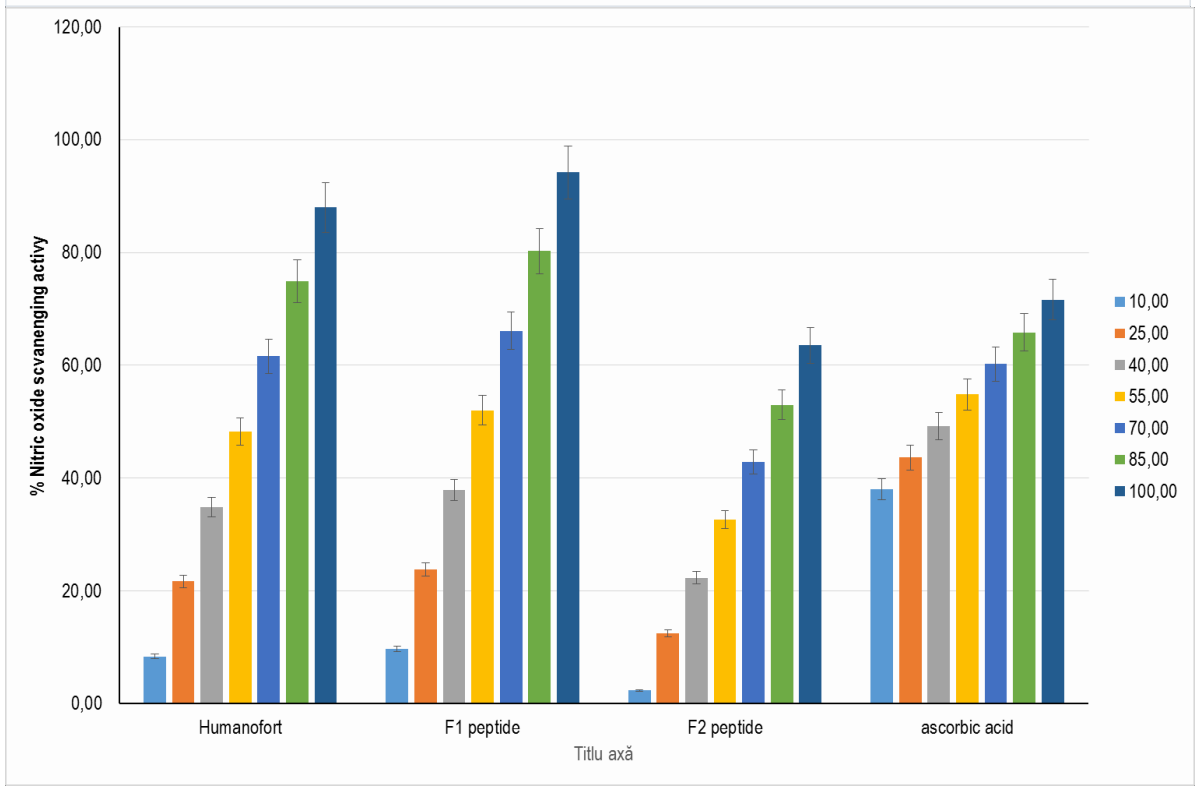
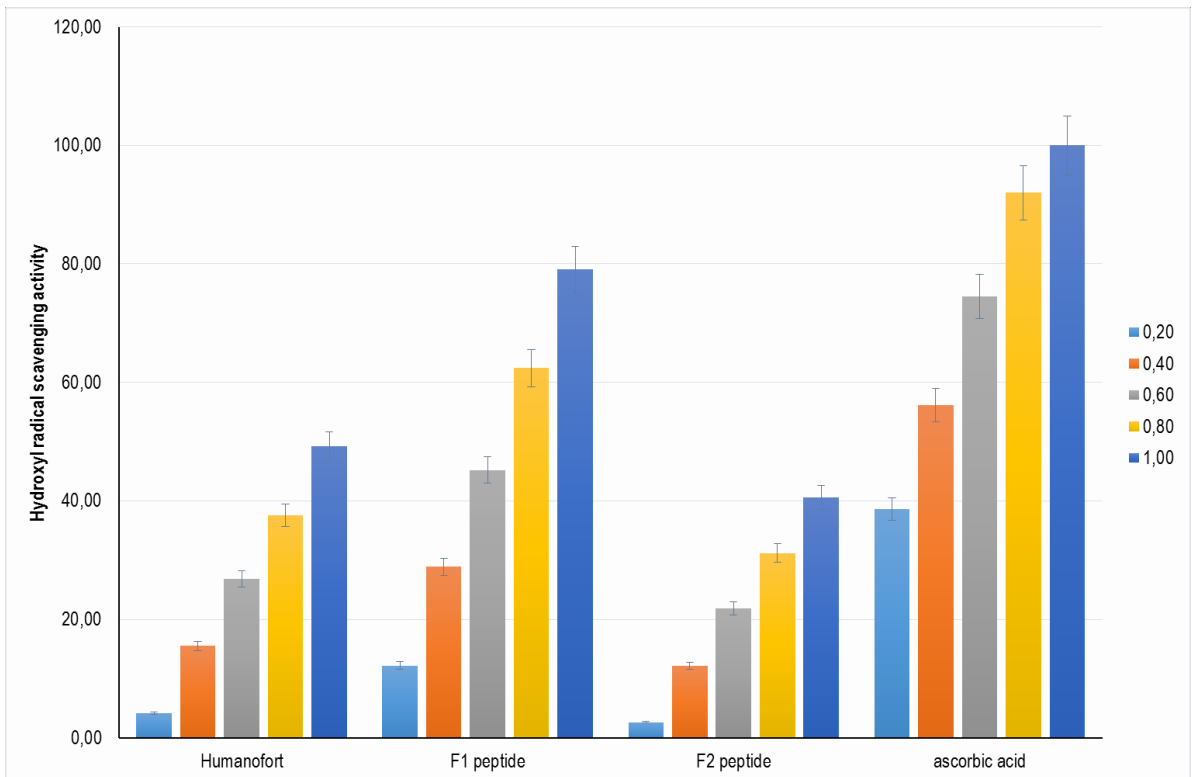
Table 1. EC50 values of Humanofort and its peptides components in different antioxidant assays

Extract/Positive Control	ABTS Radical Cation Scavenging Activity *	Superoxide Anion Radical Scavenging Activity *	Hydroxyl Radical Scavenging Activity **	Nitric Oxide Scavenging Activity *	Lipid Peroxidation Inhibitory Activity **
Humanofort	50.62 ± 0.38 ^{b,c}	42.6 ± 0,42 ^a	1.03 ± 0.01 ^{e,c}	57.63 ± 0.84 ^{e,c}	0,88 ± 0.01
F1 peptide	21.19 ± 0.17 ^{a,b}	28.42 ± 0.67 ^b	0.68 ± 0.01 ^{a,d}	52.50 ± 0.27 ^{a,e}	0,64 ± 0.01 ^{g,h}
F2 peptide	62,84 ± 0,84	ND	ND	84.2 ± 0,42	ND
Glutathione	3.25 ± 0.02 ^{c,a}	3.21 ± 0.07 ^c	NT	NT	NT
L-Ascorbic acid	NT	NT	0.11 ± 0.01 ^{f,a}	42.23 ± 0.41 ^{c,a}	NT
Trolox	NT	NT	NT	NT	17.2 ± 0.1 ^{*,h,c}

Notes: * µg/ml; ** mg/mL; ND - not determined due to low activity; NT - not tested; ^a*P* < 0.001 vs. F2 peptides ; ^b *P* < 0.001 vs. glutathione; ^c*P* < 0.001 vs. F2-peptides; ^d *P* non-significant vs. L-ascorbic acid; ^e*P* < 0.001 vs. L-ascorbic acid; ^f *P* < 0.001 vs. trolox; ^g *P* < 0.001 vs. F2-peptides.

F1 peptide was less pronounced than that of trolox (EC50 = 17.2 ± 0.1 µg/ml) (Table 1). The anti-oxidant activity of Humanofort extract and its peptide components in on the same range with that reported for chicken embryo egg hydrolysates (28). We consider this anti-oxidant activity of Humanofort and its peptides components as being relevant for the effects on probiotic bacteria.





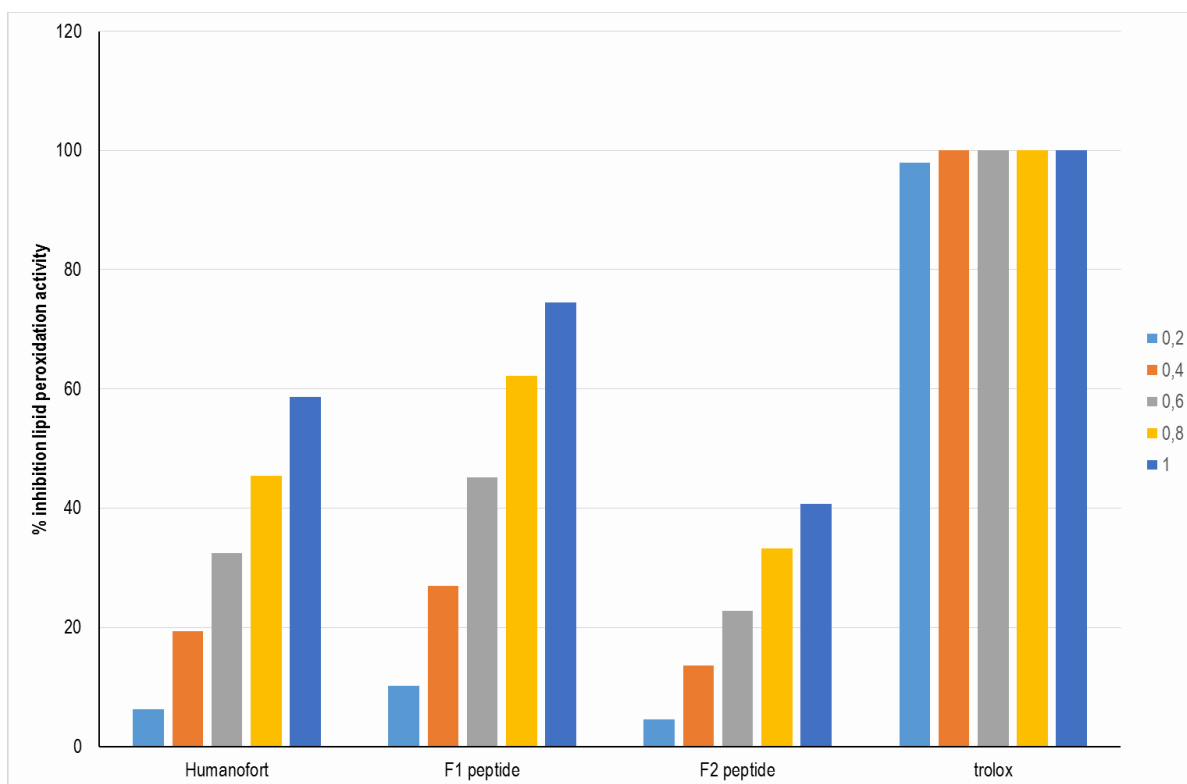


Fig. 1. (a) ABTS radical cation scavenging activity. (b) Superoxide anion radical scavenging activity. (c) Hydroxyl radical scavenging activity. (d) Nitric oxide scavenging activity. (e) Lipid peroxidation inhibitory activity.

In order to test the effects of Humanofort and its peptide components of probiotic bacteria we used probiotic bacterial strain *Lactobacillus helveticus* R0052, *Lactobacillus rhamnosus* R0011, *Bifidobacterium adolescentis* DSM 20083^T, *Bifidobacterium breve* DSM 20213^T and *Bifidobacterium longum* DSM 20219^T. The strain *Bifidobacterium longum* DSM 20219^T did not grow under aerobic conditions in any of the culture media tested. Only results obtained for the growth under anaerobic conditions are therefore presented (Table 2). Even for this strain lower values of OD_{max} and μ_{max} were obtained in un-supplemented MRS media on anaerobic conditions. Maximal growth of this strain was obtained in the media supplemented with Humanofort and its F2 peptide component. On MRS supplemented with ascorbic acid and MRS supplemented with F1 peptide, on anaerobic conditions, the growth stimulation of *B. longum* DSM 20219^T is not statistical significant.

On all other tested strains the antioxidant supplementation (ascorbic acid, Humanofort and its peptide components) stimulated the growth of tested probiotic bacterial strains on aerobic conditions. For the strain *B. breve* DSM 20213^T maximal growth under aerobic conditions, with OD_{max} and μ_{max} values of 0.34 ± 0.07 and 0.05 ± 0.02 h⁻¹, respectively, were obtained on MRS medium supplemented with F1 peptide, these growth parameters are similar with those obtained for un-supplemented

Table 2. Influence of Humanofort and its peptide components supplementation on maximum optical densities (OD_{max} , 600 nm) and maximum growth rates (μ_{max} , h^{-1}) obtained during growth, at 37 °C and under aerobic and anaerobic conditions, of several probiotic strains, in MRS media.

Bacterial strains	Aerobic conditions										Anaerobic conditions									
	MRS		MRS + ascorbic acid ¹		MRS + Humanofort ²		MRS + F1 ³		MRS + F2 ⁴		MRS		MRS + ascorbic acid ¹		MRS + Humanofort ²		MRS + F1 ³		MRS + F2 ⁴	
	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}	OD_{max}	μ_{max}
<i>L. helveticus</i> R0052	0.58± 0.11c	0.24± 0.04a	1.57± 0.12a	0.26± 0.02a	1.48± 0.21ab	0.28± 0.01a	1.59± 0.14a	0.27± 0.2a	1.35± 0.16b	0.25± 0.04a	1.47± 0.11a	0.21± 0.02b	1.53± 0.15a	0.25± 0.02ab	1.48± 0.12a	0.24± 0.04ab	1.38± 0.18a	0.19± 0.03b	1.58± 0.08a	0.28± 0.03a
<i>L. rhamnosus</i> R0011	0.46± 0.10cd	0.17± 0.02b	1.42± 0.09a	0.19± 0.02ab	1.38± 0.12a	0.18± 0.02ab	1.44± 0.08a	0.21± 0.05ab	1.16± 0.09b	0.22± 0.03ab	1.32± 0.16ab	0.20± 0.04ab	1.42± 0.12a	0.21± 0.04ab	1.34± 0.12ab	0.22± 0.04ab	1.28± 0.12ab	0.18± 0.03b	1.46± 0.09a	0.25± 0.02a
<i>B. adolescentis</i> DSM 20083 ^T	0.18± 0.09c	0.05± 0.04b	0.82± 0.11ab	0.12± 0.02ab	0.80± 0.12ab	0.10± 0.03ab	0.97± 0.09a	0.11± 0.04ab	0.67± 0.11c	0.07± 0.03b	0.52± 0.12b	0.07± 0.02b	0.92± 0.09a	0.12± 0.03ab	0.78± 0.11ab	0.11± 0.04ab	0.56± 0.14b	0.08± 0.02b	0.98± 0.10a	0.15± 0.03a
<i>B. breve</i> DSM 20213 ^T	N.G.	N.G.	0.26± 0.07ab	0.04± 0.02b	0.27± 0.08ab	0.04± 0.02b	0.34± 0.07a	0.05± 0.02b	0.17± 0.08b	0.05± 0.03b	0.28± 0.07b	0.06± 0.01b	0.47± 0.08a	0.10± 0.01a	0.38± 0.12ab	0.08± 0.03ab	0.30± 0.06b	0.06± 0.02b	0.49± 0.10a	0.11± 0.02a
<i>B. longum</i> DSM 20219 ^T	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.	0.20± 0.04c	0.04± 0.01b	0.54± 0.08a	0.10± 0.02a	0.32± 0.09b	0.06± 0.03ab	0.27± 0.08bc	0.04± 0.02b	0.56± 0.09a	0.09± 0.01a

¹-ascorbic acid (0.1%) as a positive control medium; ²-Humanofort (100 µg/ml) ³- F1 Humanofort peptide component 90µg/ml; ⁴- F2 Humanofort peptide component 10µg/ml; a,b,c,d: for a given raw, data (± standard deviation) followed by the same letter are not significantly different (P > 0.05).

MRS medium, in anaerobic conditions, but this strain did not grow aerobically on un-supplemented MRS medium. The growth of the third Bifidobacterium strain, *B. adolescentis* DSM 20083^T, was significantly stimulated by the addition of major antioxidant, Human fort, F1 peptide and ascorbic acid in anaerobic media. OD_{max} and μ_{max} values of 0.97 ± 0.09 and, respectively, 0.11 ± 0.04 h⁻¹ were obtained for this strain on aerobic conditions, on MRS media supplemented with 10 μg/ml peptide F1 from Humanofort preparation.

The *Lactobacillus* strains grew under aerobic and anaerobic conditions in all media tested. In un-supplemented MRS media, the OD_{max} value obtained was significantly lower for growth under aerobic conditions than under anaerobic conditions. Nevertheless, the growth of *L. helveticus* R0052 was greatly enhanced, under aerobic conditions, by supplementation with Humanofort and its F1 peptide component (which show a higher anti-oxidant activity), as demonstrated by high OD_{max} and μ_{max} values obtained in the aerobic media supplemented with Humanofort and, respectively, F1 peptides. These growth parameter values were similar to those obtained in un-supplemented MRS medium under anaerobic conditions and in the control medium under both conditions.

The effects under aerobic conditions are related to the anti-oxidant activity of the added components. The average initial ORP of the un-supplemented MRS medium used in this experiment was around + 249 mV. The addition of Humanofort and its peptide components F1 and F2 decreased the ORP of this culture medium within 15 min. Maximal decreases of 62.2%, 44.4% and 23.9% of the initial ORP value respectively were obtained by addition of 90μg/ml F1 peptide component, 100 μg/ml Humanofort, and, respectively 10μg/ml F2 Humanofort peptide component. Moreover, a very important decrease (331 mV) of the initial ORP value of the culture medium was obtained by the addition of 1000 μg/ml ascorbic acid and a final value of - 74.3 mV was reached.

F2 peptides, which show less significant effects on aerobic conditions, was very effective on anaerobic conditions. Its effect was very relevant especially on μ_{max} values. This fraction have also a significant effect on human fibroblast growth (12), thus the peptide growth factor present on this fraction stimulate also the development of probiotic bacteria.

Protective effects of 100 μg/ml Humanofort standardized chicken embryo extract, against the oxidative stress of *L. helveticus* R0052 grown at 37°C in MRS broth in the presence of oxygen was demonstrated also by FTIR spectroscopy. Fig. 2 presents normalized FTIR spectra, in the region characteristic of membrane components i.e., acyl chains, of *L. helveticus* R0052 cells. This strain was grown in un-supplemented MRS media in the presence or absence of oxygen or in MRS + 100 μg/ml Humanofort standardized chicken embryo, in the presence of

oxygen. This spectral region ($3000\text{--}2800\text{ cm}^{-1}$) is dominated by the CH_3 , CH_2 and CH -stretching vibrations, characteristics to the groups usually present in fatty acid components of the various membrane amphiphiles (29). The bands with frequencies near 2960 , 2930 , 2875 and 2855 cm^{-1} have been assigned, respectively, to CH_3 and CH_2 asymmetric and CH_3 and CH_2 symmetric stretching vibrations. Differences in FTIR spectra in the $3000\text{--}2800\text{ cm}^{-1}$ region for cells grown in un-supplemented media in the presence or absence of oxygen can be observed (Fig. 2). The absorption intensity of the peaks corresponding to CH_3 ($\nu_{\text{as}}\text{CH}_3$) and CH_2 ($\nu_{\text{as}}\text{CH}_2$) asymmetric stretching vibrations, obtained for cells grown in the presence of oxygen, was more important than for cells grown in the absence of oxygen. The frequency of the peak corresponding to $\nu_{\text{as}}\text{CH}_2$ stretching vibrations was also higher for cells grown in the presence of oxygen in the un-supplemented medium in comparison with cells grown in the absence of oxygen (2931.9 cm^{-1} instead of 2929.5 cm^{-1}). Spectra with intermediate peak intensities were obtained for cells grown in the presence of oxygen in MRS supplemented with $100\text{ }\mu\text{g/ml}$ Humanofort standardized chicken embryo extract.

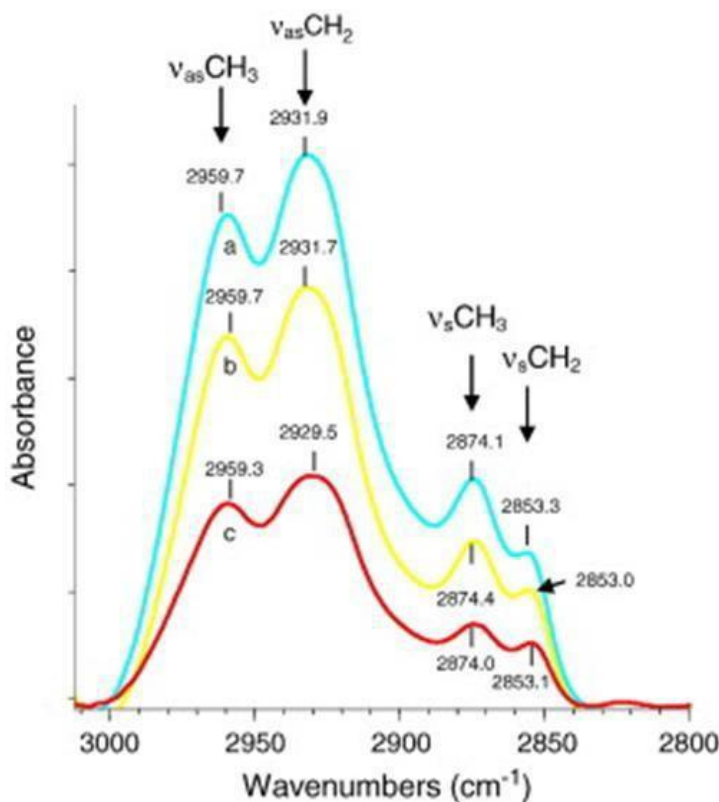


Fig. 2. Normalized FTIR spectra ($3010\text{--}2800\text{ cm}^{-1}$) of *Lactobacillus helveticus* R0052 cells grown in: (a) un-supplemented MRS media in the presence of oxygen; (b) in MRS + $100\text{ }\mu\text{g/ml}$ Humanofort standardized chicken embryo extract in the presence of oxygen and (c) in un-supplemented MRS in the absence of oxygen.

Our results shown a stimulating and protective effects of peptides from chicken embryo extract on probiotic bacteria. We separated from Humanofort, an extract from chicken embryos standardized in embryo peptides, two peptides fractions, one with a more significant anti-oxidant activity (F1) and other one which exert also a growth stimulation effect on probiotic bacteria (F2). This F2 fraction contains growth factors, with stimulating effects on human cells (12). A pre-biotic activity / stimulation of probiotic bacteria - bifidogenic activity was already demonstrated for other peptides

originating from food reach on growth factors – i.e. human milk (18) and colostrum (30).

The anti-oxidant peptides fraction protect probiotic bacterial strains from two genera, *Lactobacillus* and *Bifidobacterium*, against (nitro)oxidative stress induced by (micro)aerobic conditions. The peptides fraction which include growth factors stimulate the development of probiotic bacteria, especially under anaerobic conditions.

Probiotic bacteria, like all other gut bacteria, should face the stress of bile in order to survive in the human gastrointestinal tract (31). The mechanisms by which bile salts limit bacterial growth is related to the (nitro)oxidative stress, via disulfide stress resulted from widespread protein unfolding and aggregation caused in bacteria by bile salts (32). The antioxidant effects of Humanofort chicken embryo extract and its peptide components could be involved on *in vivo* mechanisms which lead to a better growth of probiotic bacteria.

Reactive oxygen species (ROS) and are involved in the interactions between probiotic bacteria and mammalian intestinal epithelia (33), probably via the rapid and transient oxidative inactivation of sensor proteins bearing oxidant-sensitive thiol groups. Modulation of this transient oxidative inactivation of sensor proteins by ROS generated by probiotic bacteria due to Humanofort antioxidant peptides activity could lead to a local modification of intestinal epithelia functions, including on those related to epithelial cell barrier function (34), also related to peptide absorption (including embryo-peptide absorption).

These (embryo)peptides from chicken embryo / incubated fertilized hen eggs extracts have been involved in the physiological mechanisms related to beneficial effects of ingestion of small amounts of chicken embryo / incubated fertilized eggs extracts, mainly in relation to steroidogenesis (10-12). One main hypothesis regarding the physiological mechanism of action which have been proposed is related to the influence exerted by the peptides with growth factor activity on the homeostasis of the growth factors which are controlling in an autocrine / paracrine manner the function of zona fasciculata and zona reticularis from adrenocortical cells (4). Regulation of the adrenocortical stem cell niche and postnatal maintenance of this vital population of cells is essential for proper organism functions (35). IGF-1 was demonstrated to be involved in the regulatory mechanisms controlling steroidogenesis and cholesterolemia in old subjects following the long-term administration of embryopeptides (36).

The non-absorbed peptides from such chicken embryo chicken embryo / incubated fertilized hen eggs extracts are acting also on the gut micro-flora. Their stimulating and protective effects on the beneficial probiotic bacteria, like the strains studied on this papers, could contribute to the beneficial effects revealed during the clinical trials on human subjects, wherein chicken embryo / incubated fertilized eggs

extracts were orally administrated. In the case of effects of these nutraceuticals based on chicken embryo on depression it was established a clear link between a proper function of gut microbiome and the central nervous system. The gut microbiome influences brain function and behavior (37), including on anxiety and depression (38), mainly due to the production of short fatty acids (39). This postbiotic effect - i.e. the effect related to bacterial products or metabolic byproducts from probiotic microorganisms that have biologic activity in the host (40), could be enhanced by the chicken embryo peptides with stimulating and protective function on probiotic bacteria.

Also the improvement of the metabolic syndrome described after ingestion of chicken embryo peptides could be explained by such an enhanced postbiotic effect.

Probiotic bacteria and their metabolic products attenuate the structural disruption of gut microbiota and associated inflammation involved as etiological factors in high fat diet (HFD)-induced metabolic syndrome (MS) (41). Gut microbiome (and the postbiotic products of probiotic bacteria) influence secretion of the gut peptides (orexin, galanin, ghrelin, gastrin and leptin), which were linked also with changes in sleep wake cycle, and sexual behavior (42). Galanin, for example, stimulates the activity of the central branch of the hypothalamic–pituitary–adrenal HPA axis (i.e. the release of corticotropin-releasing hormone and ACTH) and appears to play a role in modulating the HPA axis response to stress (43). Similarly, ghrelin possesses a marked ACTH / cortisol-releasing effect in humans, and is probably involved in the modulation of the HPA response to stress or changes in nutritional/metabolic status (44).

Gut microbiome could be considered as a virtual endocrine organ (45) and embryopeptide modulating activity could be linked to the beneficial effects of nutraceuticals based on chicken embryos / fertilized hen eggs extracts. Manipulating the microbial composition of the gastrointestinal tract modulates plasma concentrations of tryptophan, an essential amino acid and precursor to serotonin, a key neurotransmitter within both the enteric and central nervous systems (46). Indirectly, the gut microbiota exerts control over the hypothalamic-pituitary-adrenal axis. This is clear from studies on animals raised in a germ-free environment, who show exaggerated responses to psychological stress, which normalizes after monocolonization by certain bacterial species including *Bifidobacterium infantis* (47).

An increased body of evidence suggest a therapeutic targeting of the gut microbiota, which could be useful in treating stress-related disorders and metabolic diseases (45). Such type of disorders, stress related (including anxiety and depression) and metabolic diseases (including metabolic syndrome) are those which have been demonstrated to be improved after oral administration of different type of preparation made with chicken embryo / fertilized hen eggs extracts. The effects of embryopeptides from Humanofort extract on probiotic bacteria support this putative link.

We proposed two different physiological mechanisms involved into beneficial effects on human health of orally ingested chicken embryo / partially incubated hen

eggs: a direct one, resulted from the intestinal absorption of peptides with anti-oxidant and growth factors activity, and an in-direct, mediated one, due to an enhanced post-biotic effect of probiotic microbiome – fig. 3. These mechanisms are inter-connected, peptide absorption and further expression of their biological activity, being mediated by the interaction with the gut epithelium and microbiota (16).

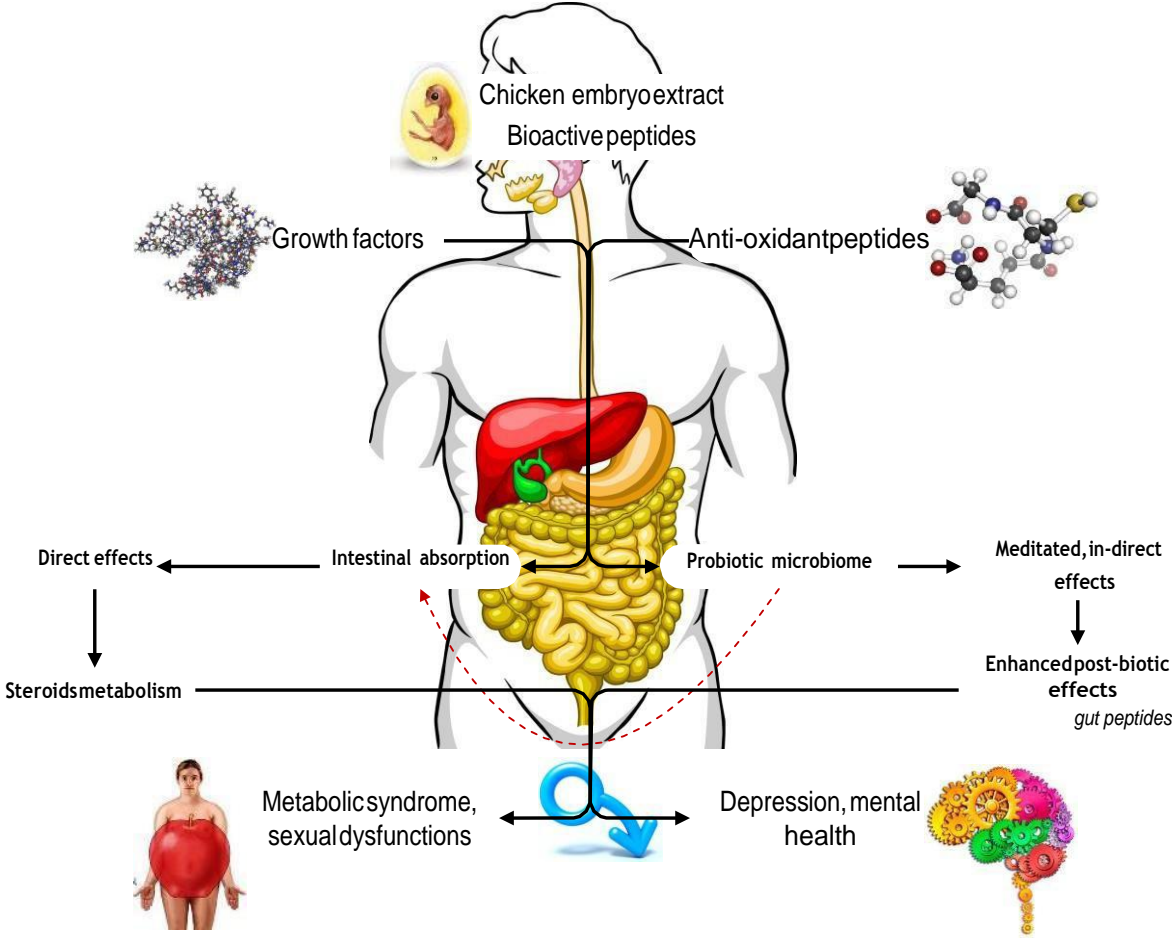


Fig. 3. Physiological mechanisms involved into beneficial effects on human health of orally ingested chicken embryo / partially incubated hen eggs: a direct one, resulted from the intestinal absorption of peptides with anti-oxidant and growth factors activity, and an in-direct, mediated one, due to an enhanced post-biotic effect of probiotic microbiome

Peptide with growth factor activity could modulate also the local response of the gut epithelium and of the gut microbiome, including the production of gut peptides or absorption of metabolic byproducts of probiotic bacteria related to post-biotic effects (e.g. short fatty acids). Further studies are necessary in order to further provide evidences of the indirect, gut microbiome mediated effect of chicken embryo / fertilized hen eggs extracts

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