

USE OF PROBIOTICS FOR IMPROVING PROTEIN ABSORPTION

Technical field

The present invention relates to the use of probiotics, preferably one or more probiotic bacteria, to increase the absorption of proteins or the
5 bioavailability thereof, preferably in individuals with increased protein and/or energy requirements, preferably elderly persons, children, pregnant women or athletes.

State of the Art

Proteins are an essential part of living organisms and are formed by the
10 union of simpler molecules called amino acids that bind together through peptide bonds.

In the human body about 50,000 different protein molecules are present; their function is determined by their amino acid sequence. Through a series of reactions, our body is capable of autonomously synthesising the
15 proteins it needs from the single amino acids contained in foods.

Many proteins belong to the category of enzymes, whose function is to catalyse the biochemical reactions that are vital for the metabolism of organisms.

Some have structural and mechanical functions, like actin and myosin in
20 muscles, collagen in bones and tissues, and as components of the cell cytoskeleton.

Other proteins are important mediators in the transmission of inter- and intracellular signals, in the immune response, in cell adhesion mechanisms and in the cell division cycle.

25 Since proteins cannot be absorbed as such and transported in the circulation, several enzymes present in the lumen of the gastrointestinal tract intervene in their digestion, breaking them down into single amino acids.

During the digestive process, the majority of proteins are completely
30 reduced into single amino acids.

The digestion of these macromolecules begins in the stomach, where the combined action of pepsinogen and hydrochloric acid leads to the formation of oligopeptides (short amino acids chains of less than ten units).

- 5 This digestion is then completed by the intestinal proteases of pancreatic origin (dumped into the duodenum) and produced by the membranes of the intestine itself (situated on the brush border). For this reason, protein digestion is normal even after the surgical removal of the stomach.

The above-mentioned proteases are divided into endoproteases (that
10 hydrolyse the internal peptide bonds of proteins: chemotripsin, elastase, tripsin) and exopeptidases (that hydrolyse the terminal amino acid of the protein: carboxypeptidase, aminopeptidase, dipeptidase).

Protein digestion is completed at the intestinal level, thanks precisely to the action of exopeptidases, present in the microvilli of the small intestine,
15 which lead to the formation of single amino acids, dipeptides and tripeptides, which can thus be absorbed at the site of the mucosa via a Na^+ or H^+ symport mechanism.

Moreover, intestinal flora present in the small intestine, above all lactobacilli, contribute to further digesting the peptides by acting also on
20 the ones not completely hydrolysed by the proteases themselves. Only a small portion, equal to about 5%, arrives in the colon, where it undergoes as well the action of the resident bacterial flora (lactobacilli and bifidobacteria).

Once absorbed, the single amino acids are transported to the liver by
25 specific carriers and here they can:

- be used as such and intervene in the immune response, in the synthesis of hormones and vitamins, in the transmission of nerve impulses, in the production of energy and as catalysts in many metabolic processes;

- participate in protein synthesis, an inverse process in respect to the digestive one which has the purpose of providing the body with materials for the growth, maintenance and reconstruction of cellular structures;
- if present in excess, be used for energy purposes (gluconeogenesis) or converted into fat deposits.

A small portion of proteins present in foods, however, is not absorbed and is eliminated as such with faeces (5%).

Some peptides made up of more than three amino acids are absorbed by transcytosis and as such they can represent a significant element for the development of allergies and food intolerances.

The human body breaks down proteins daily while synthesising others. This process is defined as protein turnover. In this process, some amino acids are oxidated, and the resulting nitrogen is lost in the form of urea, creatinine and other derived substances. With a normal protein intake, only 4% of the proteins turned over may be lost.

This situation can be determined by the amount of protein that is ingested, i.e. by a high or a low daily protein intake. The nitrogen is mainly dispersed through urine, but a part is also eliminated through sweat, faeces, skin or nails.

The nitrogen balance compares the amount of nitrogen (from dietary proteins) introduced into the body with the nitrogen that is lost. If an individual takes in more nitrogen than he/she loses, he/she is said to have a positive nitrogen balance and deposits nitrogen in the body. If an individual consumes the same amount of nitrogen as he/she loses, he/she is said to be in a situation of nitrogen equilibrium, whereas if an individual loses more nitrogen than he/she consumes, he/she has a negative nitrogen balance and loses body proteins.

Since catabolism or the amino acids breakdown is the principal cause of nitrogen loss, the expulsion of nitrogen is an indicator of the amino acids catabolism.

Protein requirements are defined by the amount of dietary proteins necessary to compensate for and offset the loss of nitrogen on a daily basis, so that a person maintains a nitrogen balance. This is determined by measuring the excretion of nitrogen when the person follows a protein-free diet. Since the intake of dietary nitrogen is equal to zero, all of the nitrogen expelled originates from the breakdown and catabolism of body proteins. As mentioned, this value presumes the presence of sufficient food calories and a normal proportion of food carbohydrates.

When we refer to a normal healthy body, we exclude situations in which the nitrogen balance can be altered, such as pregnancy, breastfeeding, old age, sports activity, convalescence, growth (children, adolescents), or the aftermath of a low-calorie diet or anorexia.

Protein requirements are the amount of proteins our body needs to satisfy its energy needs and maintain good health.

These amounts vary according to several factors, such as: age, gender, state of health, work activity or sports activity.

The average protein requirements of a person are inversely proportional to age. For example: about 2 g/kg/day in newborns, about 1.5 g/kg/day at 5 years, and about 1.2 g/kg/day in adolescence – adulthood.

A protein is digestible if a high proportion of its amino acids reach the body's cells so that they can synthesise the proteins they need.

Not all sources, however, are used equally well by the body and the bioavailability of proteins themselves varies according to the protein source. In fact, nearly 100% of animal proteins undergo intestinal absorption, whereas vegetable proteins show a much lower absorption: 52% for lentils, 70% for chick peas and 36% for wheat.

In order to increase protein bioavailability, the body breaks down the large amounts of protein molecules into their simpler units, amino acids.

In adults, a protein deficiency causes a loss of vigour and strength, mental depression, profound weakness, poor resistance to infections, delayed

scarring of wounds and slow recovery from illnesses. Even hair and nails are affected, and this problem is more serious for children.

As pointed out, in order for the body to be able to use proteins, they must be broken down into free amino acids or peptides. The proteins in natural yogurt containing live lactobacilli arrive at the digestion process in already pre-treated conditions. Indeed, even before it is eaten, the bacteria present have begun to transform the proteins, fats and sugars of the milk into easily digestible components. As a result, the nutrients in natural yogurt are immediately available and ready for use by our body.

- 10 The fermentation process measurably increases the protein content of fermented foods. Even protein-rich foods like milk contain more proteins after fermentation than before the bacteria interacted with them. When we eat a fermented food like yogurt, not only do we have the advantages of the added proteins, but this food in a partially pre-digested, completely bioavailable form is also very appreciated by the body.

Object of the invention

- With the aim of satisfying, in general, the average daily protein requirements and particularly in conditions in which the nitrogen balance is altered, the present invention proposes using one or more probiotics in order to increase protein bioavailability in the body or in any case the absorption (assimilation) thereof, preferably at the intestinal site.

Detailed description of preferred embodiments of the invention

- A first aspect of the present invention relates to the use of probiotics for increasing the absorption and/or the bioavailability of proteins and/or of protein derivatives in an individual.

- Preferably, the individual has a need for an increased protein absorption/bioavailability, for example as in the case of elderly persons, children, pregnant women or athletes. In other words, said probiotics can be taken in order to increase the protein and/or energy intake every time the need arises for an individual.

In this context “probiotic” means, according to what has been established by the FAO and WHO: “*Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host*”.

In other words, probiotics are microorganisms which demonstrate to be able, once ingested in adequate amounts, to perform functions that are beneficial for the body, substantially echoing the definition of the two above-mentioned organisations.

In the context of the present invention, protein requirements means the amount of proteins our body needs to satisfy its energy needs and maintain good health.

These amounts vary according to several factors, such as: age, gender, state of health, work activity or sports activity.

The average protein requirements of a person are inversely proportional to age. For example: about 2 g/kg/day in newborns, about 1.5 g/kg/day at 5 years, and about 1.2 g/kg/day in adolescence – adulthood

Preferably, the proteins to which reference is particularly made in the context of the present invention are selected from among animal and vegetable proteins, including: egg white, milk serum proteins, preferably WPI 90% and WPC 80%; lupin proteins, preferably protilup 450; soy proteins; pea proteins and caseinate protein, preferably calcium caseinate protein and rice proteins.

In fact, the applicant has surprisingly found that following the administration of probiotics, in particular of bacteria of the genus *Lactobacillus*, preferably the strains L. casei DG® (*Lactobacillus paracasei* CNCM I-1572) and/or *Lactobacillus paracasei* LPC-S01, one observes a 10-20% increase in the protein/energy absorption/bioavailability in the individual who has taken them. In particular, the effect has been observed for caseinate and/or milk serum proteins.

According to a preferred embodiment of the present invention, the probiotics are bacteria and/or yeast and/or other microorganisms, taken individually or in combination.

The bacteria that are particularly preferred for the purposes of the present invention belong to a genus selected from among: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Aerococcus*, *Enterococcus* and combinations thereof.

- 5 More preferably, the bacteria belong to the genus *Lactobacillus* and/or *Bifidobacterium*.

In particular, the *Lactobacillus* is selected from among: *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus alimentarius*, *Lactobacillus*
 10 *aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*,
 15 *Lactobacillus johnsonii*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefir*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus collinoides*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius*, *Lactobacillus*
 20 *sanfranciscensis* and combinations thereof.

More preferably, the lactobacilli are of the species *Lactobacillus acidophilus*, *rhamnosus* or *paracasei*. Even more preferably, the strains L. casei DG® (*Lactobacillus paracasei* CNCM I-1572) and/or *Lactobacillus paracasei* LPC-S01 (deposited by SOFAR S.p.A. with the German
 25 Collection of Microorganisms of the institute DSMZ of Leibniz on 16/01/2016, with deposit number DSM 26760) are used. They can be administered individually or in various combinations.

The bacterial strain L. casei DG® was deposited by SOFAR S.p.A. with the National Collection of Microorganism Cultures of the Pasteur Institute of
 30 Paris on 05/05/1995, with deposit number CNCM I-1572. Initially, the strain had the designation *Lactobacillus casei* DG sub.casei.

Clearly, use can also be made of other bacteria that are capable of stably colonising the intestine, taking away space from pathogenic bacteria.

The amount of microorganism administered, in particular for the bacteria, is the minimum sufficient to obtain a temporary colonisation of the intestine, preferably at least 10^9 units of microorganism, bacterium, per day.

The microorganisms, preferably the bacteria, can be administered live and therefore the composition is also definable as a probiotic. Alternatively, the microorganisms that can be used are dead or tyndallized.

In a further embodiment, the microorganisms are in the form of a lysate or extract and therefore the composition is also definable as a paraprobiotic, or a single component, or several components thereof, present at the level of the bacterial wall.

In a further embodiment of the invention, the composition further comprises the metabolic bioproducts generated by the microorganisms defined as postbiotics, and/or any other product of bacterial derivation. Therefore, the composition of the present invention is also a known or presumed probiotic or paraprobiotic or postbiotic or a component of the bacterial wall.

In general, the microorganisms comprised in the composition of the present invention are single microorganisms or combinations of any microbial species specified in the QPS list of the EFSA.

In one embodiment of the present invention, the probiotics can be administered orally, for example in solid form, preferably as pills, capsules, tablets, granular powder, hard-shelled capsules, orally dissolving granules, sachets, lozenges or drinkable vials.

Alternatively, in liquid form, for example as a syrup or beverage, or else they are added to food, for example to yogurt, cheese or fruit juice.

Alternatively, they are formulated in a form capable of exerting a topical action, for example via enema or as a cream.

They can also be administered in combination with amino acids, supplements, vitamins, trace elements such as zinc and selenium, enzymes and/or prebiotic substances, such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, guar gum or combinations thereof.

The Applicant has found that probiotics in general, and in particular bacteria of the genus *Lactobacillus* and/or *Bifidobacterium*, are very effective in splitting proteins into different peptides compared to what occurs by virtue of the action of digestive enzymes alone. They also act upon molecules that are not completely hydrolysed by human proteases, but whose hydrolysis could be completed by microbial intervention.

The peptides generated by virtue of their action, individual or combined, are more easily absorbed by the body than the starting protein. The final result that is obtained by the action of the probiotics is an increased/improved absorption or, in any case, a greater bioavailability of the proteins to an individual who needs them.

EXAMPLE I

In particular, an in vitro study was set up for the purpose of evaluating the role of probiotic bacteria in protein absorption; in particular, the proteins used by athletes were tested.

The probiotic bacteria used specifically by way of illustrative example are:

- L. casei DG® (*Lactobacillus paracasei* CNCM I1572); and
- *Lactobacillus paracasei* LPC-S01.

The bacteria were used individually or in combination.

The individual or combined action was verified on various protein substrates. In particular on:

- Egg white
- Milk serum proteins, WPI 90% and WPC 80%
- Lupin proteins, Protilup 450
- Soy proteins
- Pea proteins

- Calcium caseinate
- Rice proteins

As an indirect assay for evaluating the action of a protease, the overall proteolysis was evaluated by measuring absorbance at 280 nm.

This procedure is based on the quantification of soluble peptides after treatment with TCA so as to determine absorbance at 280 nm, with respect to aromatic amino acids (Phe, Tyr, Trp). Obviously, this method gives a relative indication, as it is based on the assumption that there is an average distribution of peptides containing these residues. It shall be noted that there is a direct proportionality between absorbance values and the concentration of the solution considered. Therefore, in this case high absorbance values indicate high concentrations of the peptides that are generated due to the action of the proteases and of the tested strains. However, this technique has a limit in that very small peptides are not detected and therefore the effect can actually be underestimated.

By way of example, in Table I we present the results obtained with milk serum proteins WPI. They show differences of about 10-20%.

Table I

Treatment	WPI
Pepsin	0.80 ±0.048
pepsin + pan	2.29 ±0.180
pep + pan + SO1	2.81 ±0.191
pep + pan + DG	2.45 ±0.192
Complete mix	2.51 ±0.141

Furthermore, the TCA-soluble peptides generated by the proteolytic action on caseinate with and without probiotics were also quantified.

Specifically, the peptides which showed to be soluble after treatment with TCA were separated by RP-HPLC.

The results obtained from the chromatographic separations – expressed in terms of the total area subtended by the chromatogram with 220 nm

detection – are presented in Table II below.

Table II

Proteolysis conditions	Caseinate Substrate	
	Area (*10 ⁶)	Δ
pep + pan	303	
pep + pan + SO1	322	+ 6%
pep + pan + DG	329	+ 9%

The values shown were calculated from the HPLC plots. In particular, the total area subtended by the peaks related to the peptides produced during the hydrolysis tests under the different conditions used in the assay is shown and the percentage variations relative to the treatment with pepsin and pancreatin (but without microorganisms) are indicated for the various samples.

The results show an increase in proteolysis in the presence of both microorganisms.

As said previously, the proteolytic activity measured in this manner could however be underestimated due to the production of very small protein fragments that do not adhere to the column matrix and are thus lost in the fraction eluted prior to the application of the chromatographic gradient and which are consequently not quantifiable. Or else it could also occur that the use of probiotics increases the proteolytic action in such a way as to fragment the proteins into peptides that are so small as to be immediately eluted from the column.

EXAMPLE II

The proteolysis tests were conducted using the strain L casei SO1 or DG individually.

The substrates used were:

- milk serum proteins, WPI 90%, batch R2522, supplied by SOFAR
- isolated rice proteins supplied by SOFAR,
- concentrated serum proteins, WPC 80%, supplied by SOFAR
- pea proteins, batch 20150430, supplied by SOFAR

5 Evaluation of total proteolysis by measuring absorbance at 280 nm

The conditions of proteolytic activity are the same as used in the preceding experimental tests.

By way of example we show the results obtained in Table III.

Table III

Treatment	WPI	WPC	Rice
pep + pan	2.45	2.11	2.06
pep + pan + SO1	2.48	2.12	2.09
pep + pan + DG	2.44	2.09	2.07
pep + pan + mix	2.50	2.11	2.11

10 Evaluation of total proteolysis by RP-HPLC separation

With this method we wanted to quantify the peptides generated by the proteolytic action. Specifically, the peptides showing to be soluble after treatment with TFA 0.1% – which constitutes the mobile phase used for HPLC separation – were separated by RP-HPLC. Under these conditions,
 15 in fact, most of the protein material that is separable and quantifiable by measuring the absorbance at both 220 nm and 280 nm remains soluble.

The results obtained from the chromatographic separations – expressed in terms of the total area subtended by the chromatogram with detection at 220 nm or at 280 nm – are presented in Table IV. It should be observed
 20 that the quantification at 220 nm allows to observe also the presence of peptides that do not contain the aromatic residues (Phe, Tyr, Trp), which are instead quantified when absorbance is measured at 280 nm.

The values shown were calculated from the HPLC plots. In particular, the total area subtended by the peaks related to the peptides produced during
 25 the hydrolysis tests under the different conditions used in the assay is

shown, and the percentage variations relative to the treatment with pepsin and pancreatin (but without microorganisms) are indicated for the various samples.

The results presented in Tables IV and V show that the L.casei DG and S01 strains have a comparable proteolytic activity with respect to the serum protein substrate. In the case of rice proteins, on the other hand, it should be noted that only the L.casei S01 strain shows a proteolytic activity. The L.casei DG strain, by contrast, is more active with respect to the pea protein substrate.

10 Table IV

Treatment	Substrate							
	WPI		WPC		rice		pea	
	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ
pep + pan	125		98		78.2		107	
pep + pan + SO1	128	+ 3%	101	+ 3%	80.2	+ 3%	107	-
pep + pan + DG	131	+ 5%	102	+ 4%	78.4	-	111	+ 4%
pep + pan + mix	129	+ 3%	104	+ 6%	80.6	+ 3%	114	+ 7%

Table V

Treatment	Substrate							
	WPI		WPC		rice		pea	
	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ	Area (*10 ⁶)	Δ
pep + pan	4.5		3.6		5.1		11.9	
pep + pan + SO1	4.9	+ 9%	3.9	+ 8%	5.4	+ 6%	12.0	-
pep + pan + DG	4.8	+ 7%	3.8	+ 6%	5.2	+ 2%	12.2	+ 3%
pep + pan + mix	4.9	+ 9%	3.9	+ 8%	5.4	+ 6%	12.6	+ 6%

CLAIMS

1. Use of probiotics for increasing the absorption and/or the bioavailability of proteins and/or of energy in an individual.
2. The use according to claim 1, wherein said probiotics are selected from: bacteria, yeasts and other microorganisms, taken singularly or in combination; preferably the bacteria belong to at least one genus selected from: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Streptococcus*, *Lactococcus*, *Aerococcus* and *Enterococcus*.
3. The use according to claim 1 or 2, wherein said bacteria belonging to the genus *Lactobacillus* are selected from: *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus alimentarius*, *Lactobacillus aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus johnsonii*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefiri*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus collinoides*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius* and *Lactobacillus sanfranciscensis*, preferably selected from: *Lactobacillus acidophilus*, *rhamnosus* or *paracasei*.
4. The use according to any one of claims 1-3, wherein said bacteria belonging to the genus *Lactobacillus* are selected from *L. casei* DG® (*Lactobacillus paracasei* CNCM I-1572) and/or *Lactobacillus paracasei* LPC-S01 (DSM 26760 DSMZ) strains.

5. The use according to any one of claims 1-4, wherein said probiotics are taken daily by said individual in an amount sufficient to allow temporary gut colonization, preferably at least 10^9 units of microorganism, preferably of bacterium, per day.
- 5 6. The use according to any one of claims 1-5, wherein said individual is selected from: the elderly, children, pregnant women and athletes.
7. The use according to any one of claims 1-6, wherein the proteins, animal and/or vegetable, are selected from: egg white, milk serum
10 proteins, preferably WPI 90%; lupin proteins, preferably protilup 450; soy proteins; pea proteins; caseinate protein, preferably calcium caseinate protein and rice proteins.
8. The use according to any one of claims 1-7, wherein the probiotics, preferably the bacteria, are administered live, preferably by the oral
15 route, more preferably in solid form, even more preferably as pills, capsules, tablets, granular powder, hard-shelled capsules, soluble granules, sachets or lozenges; or as a liquid, preferably as a syrup or beverage, or they are added to food, preferably yogurt, cheese or fruit juice.
- 20 9. The use according to any one of claims 1-8, wherein the probiotics are formulated to act topically, preferably via enema or as a cream.
10. The use according to any one of claims 1-9, wherein the probiotics are administered in combination with amino acids, supplements, vitamins, trace elements, preferably zinc and/or selenium, enzymes
25 and/or prebiotic substances, preferably fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, guar gum or a combination thereof.

ABSTRACT

The present invention relates to the use of probiotics, preferably one or more probiotic bacteria, to increase the absorption of proteins or the bioavailability thereof, preferably in individuals with increased protein
5 and/or energy requirements, preferably elderly persons, children, pregnant women or athletes.