

PROBIOTICS AND ORAL TOLERANCE

Background of the invention

5 Cow's milk allergy or milk hypersensitivity is very common in infants and usually disappears by the age of two or three years, but may occasionally be lifelong. It is the most common disease in infants, with an incidence of 0.5 to 3% in full term infants and 3 to 5% in preterm infants. This allergy can cause rash, hives, redness around the mouth, runny nose, sneezing, colic, diarrhoea, vomiting, anaphylaxis, or more generally
10 digestive troubles. It could also be associated in some cases of infant sudden death.

Milk hypersensitivity should be differentiated from lactose intolerance, which is an intolerance to milk as a result of congenital deficiency of the lactase enzyme.

15 Cow's milk allergy is caused, in most cases, by the α -lactoglobulin and the β -lactoglobulin allergens. It can also be caused by casein and/or albumin, which are potentially allergenic lactic proteins also present in cow's milk. The allergy, when developed, is caused by an hypersensitivity reaction of the immune system to the above-mentioned proteins. In the first step of the process (sensitisation), the immune system
20 recognises the protein as an antigen when it enters the body, and this generates an immune response consisting of specific antibodies or specifically sensitised T lymphocytes. In the second step, if the antibody is an IgE antibody, it will respond to the presence of the allergen by generating an inflammatory reaction, which is the allergy.

25 The mechanism of this type of allergy can be explained as follows: the IgE antibodies appear on the surface of cells, including circulating basophils. When the interaction allergen/IgE occurs, the cells presenting the IgE/allergen couple generates and releases chemical mediators, including histamine. This phenomenon leads in pathologic effects, such as vasodilatation, locally or systemically.

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Usually, this milk hypersensitivity appears with the first attempt of food diversification when the infant is first presented to the cow's milk.

WO 01/97822 to Oy aboatech AB discloses the use of a probiotic bacteria, for example
35 *Lactobacillus casei* ssp. *rhannosus*, for a preparation of a composition useful for primary prevention of atopic diseases in an infant at high risk of atopic diseases. It is an attempt to prevent allergies in a specific population which is the high risks of allergy population.

WO 01/89541 to Compagnie Gervais Danone discloses the use of a *Lactobacillus casei* in a composition for oral administration to enhance immunity specific to pathogenic micro-organisms. It is particularly intended for pathogens affected respiratory system, and the composition can be a food or a food supplement.

However, a need exists for a mean of preventing atopic diseases such as cow's milk allergies and/or maintaining the oral tolerance acquired by the infant, either by himself or with the help of said aforementioned mean.

Summary of the invention

Accordingly, the present invention provides the use of a *Bifidobacterium* strain for the manufacture of a food product for the maintenance of oral tolerance to antigens. The invention further provides the use of a *Lactobacillus* and a *Bifidobacteria* for the manufacture of a food product for the promotion and maintenance of oral tolerance to antigens.

The invention refers to food products which are not intended for allergic populations.

Figures

Fig 1 shows the decrease of BLG-specific antibody titers (IgE, IgG1 and IgG2a) in serum of mice (conventional (CV), monoassociated and germfree (GF)) killed 28 days (A) or 50 days (B) after oral feeding with whey proteins

Fig 2 shows the production of IFN- γ , IL-10, IL-5 and IL-4 of spleen cells in vitro. Mice (Conventional (Conv), monoassociated and germfree (GF)) were orally fed whey proteins (black bars) or saline water (open bars) before being subcutaneously injected with 100 μ g of BLG and 100 μ g of OVA in Al(OH)₃ and sacrificed 28 days (A) or 50 days (B) after gavage. Mice sacrificed at day 50 received two additional injections at day 21 and 35..

Detailed description of the invention

In the context of the present invention, the term "food product" is intended to encompass any consumable matter. Hence, it may be a product intended for the consumption by humans, but the term also encompasses products to be consumed by

animals, for example pets, such as dogs, cats, rabbits, guinea pigs, mice, rats, birds (for example parrots), reptiles and fish (for example goldfish). However, the term also includes food to be consumed other domesticated animals, such as livestock, for example, cattle, horses, pigs, sheep, goats, buffaloes, camels, and the like. This term is also intended to include any infant formula, baby formula, infant and baby follow-up formula, and the like.

It has to be understood that when talking about cow's milk allergy or cow's milk hypersensitivity, we mean any food allergy or hypersensitivity, and more generally any atopic disease. Our argumentation is mostly developed around this allergy because cow's milk is in most cases the first food product encountered by infants; however, we do not limit the scope of the present patent application to this disease.

Surprisingly, we have found that the addition of probiotics to food products could be used as an adjuvant to promote oral tolerance, and to maintain it. Specifically, we have found that lactic acid bacteria strains belonging to the *Lactobacillus* genus, and in particular *Lactobacillus paracasei* strains are able to promote the induction of oral tolerance, and is particularly suitable to promote the oral tolerance to cow's milk proteins.

In a preferred embodiment of the invention, the *Lactobacillus* used is *Lactobacillus paracasei* CNCM I-2116. These microorganisms have been shown to exhibit inter alia the following properties: they are gram positive, catalase negative, NH_3 from arginine negative, and CO_2 production negative. They produce L(+) lactic acid and are capable to grow in the presence of bile salts in a concentration of up to 0.4%.

According to the invention, the lactic acid bacteria used can be added as an adjuvant or a supplement in particular to infant formulas used from birth to the introduction of antigens (such as cow's milk antigens) to the alimentation, in order to promote the oral tolerance to the said antigens. As the lactic acid bacteria will not be a dominant flora of the infant's intestine, large amounts of the microorganism should be incorporated to the formulae, for example 10^5 to 10^8 cfu/mL of reconstituted formulae or cfu/g of food product, more preferably 10^5 to 10^7 , and in a preferred embodiment 10^6 cfu/mL of reconstituted formula. Thus, the lactic acid bacteria will be ingested several times a day, leading to a quantity of this micro-organism in the gut microflora constantly sufficient to be effective for the purpose of the present invention.

The aforementioned lactic acid bacteria, belonging to the *Lactobacillus* genus, being in particular a *Lactobacillus paracasei* and in a preferred embodiment *Lactobacillus paracasei* CNCM I-2116 can also be incorporated in pet food products in order to

promote oral tolerance. It can be incorporated in chunk products or croquettes preferably, but it can also be incorporated in humid products, such as cans, for example. It can also be added to liquid formulas for pets, such as milk for kittens or puppies, among others, and can as well be added to chew products for pets.

- 5 The supplementation of pet food by this micro-organism can be comprised, for example, between 10^4 and 10^8 cfu/g for non humid products, 10^5 to 10^8 cfu/g for humid products, and 10^5 to 10^8 cfu/g for liquid products, these amounts not being limitative.

We further investigated the proteolytic activity of *Lactobacillus paracasei* CNCM I-2116 and its potential role in the degradation of the most important allergen in milk: β -lactoglobulin.

Proteolytic system of lactic acid bacteria can be divided into three groups on the basis of their function:

- 15 i) proteinases that breakdown whole protein to peptides,
ii) peptidases that degrade peptides and
iii) transport systems that translocate the breakdown products across the cytoplasmic membrane.

Proteases are present outside the bacterial cells, whereas most of peptidases are found in the cytoplasm. In the study, peptidases from cytoplasmic extract have been used to
20 degrade native β -lactoglobulin (BLG) and its tryptic-chymotryptic (TC) peptides. *L. paracasei*-associated enzymes hydrolyze mainly acidic TC peptides while basic peptides are slightly degraded and BLG not at all. Since, BLG TC hydrolysate was shown to be composed mainly of acidic peptides (62%), their degradation with *L.*
25 *paracasei* enzymes is of great interest. However, the proteolytic activity is certainly limited to amino- or di-peptidases because *L. paracasei* degradation releases peptides with molecular weights mainly lower 500 Da, as observed by size exclusion chromatography.

The basic fraction is also degraded by amino- and di-peptidase activities and we have
30 also observed that peptides with molecular weights higher than 2000 Da appear in the fraction after *L. paracasei* degradation. Although not wishing to be bound by theory, strong hydrophobic interactions between peptides could explain this observation because basic TC peptides have been shown to be highly hydrophobic by C18 chromatography.

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The degradation of acidic TC peptides with *L. paracasei* extract lead to peptides with immunosuppressive properties, these effects being essential for oral tolerance induction. Well-characterised mechanisms for the induction of tolerance include clonal deletion,

clonal anergy and active suppression via the induction of regulatory T cells. The type 1-regulatory T cells (Tr-1) have a low proliferation capacity and suppress naïve and memory T helper type 1 and 2 responses due to their ability to produce high levels of immunosuppressive cytokines like IL-10. IL-10 was found to downregulate CD54
5 expression, CD80 and CD86 which function as important costimulatory molecules for T cell activation. Interestingly IL-10 was found to be up-regulated in response to *L. paracasei*-degraded acidic peptides indicating an effect of these peptides to induce oral tolerance to BLG by a mechanism of active suppression.

10 The potential beneficial effect of *L. paracasei*-degraded acidic peptides on oral tolerance phenomenon, underlined in BLG-naïve mice, is reinforced by the results obtained in BLG-primed mice (BLG-tolerant mice). A suppression of lymphocyte proliferation has also been observed with splenocytes from BLG-primed mice indicating a real capability of degraded peptides to induce, maintain and reinforce
15 hyporesponsiveness of T cells.

L. paracasei CNCM I-2116 stimulates regulatory T cells through its proteolytic activity and liberation of bioactive peptides. We have also demonstrated an immunosuppressive effect of the cytoplasmic content of *L. paracasei*. These findings strongly suggest that
20 bacteria such as *L. paracasei* CNCM I-2116 induce an immunosuppressive activity, either directly via cell to cell interactions or via degradation of antigens and liberation of cytoplasmic content after death.

We have also discovered that strains of the Bifidobacterium genus have a specific effect
25 on the oral tolerance maintenance. Consequently, it is a purpose of the invention to add Bifidobacteria to foodstuffs as a supplement or an adjuvant, especially to populations wherein oral tolerance has already been induced. In particular, such population can be a breast-fed infant population; indeed, part of the allergens eaten by the mother are given to the child via the mother's milk; in this population, oral tolerance has been promoted
30 via breast-feeding, and this tolerance can be maintained by giving to the infant an effective amount of Bifidobacteria having the aforementioned property.

It is well known that some infants, who do not develop cow's milk hypersensitivity when they first encounter cow's milk, nevertheless develop some food hypersensitivity when they encounter other food ingredients, for example at the age of 8 months.
35 Although not wishing to be bound by theory, we believe that they have induced an oral tolerance which has not been maintained or not been maintained properly.

Consequently, a purpose of the invention is to maintain an oral tolerance previously induced, by means of adding to the alimentation a Bifidobacteria having the ability to do so.

5 It might be useful to give the bifidus to the child or the infant at the beginning of the process.

In a preferred embodiment of the invention, the Bifidobacteria is a *Bifidobacterium lactis*, and can be in the best mode of realisation the well-known Bb12 strain, ATCC 27536 which can be obtained from Hansen (Chr. Hansen A/S, 10-12 Boege Alle,
10 P.O.Box 407, DK-2970 Hoersholm, Danemark). As the nomenclature has changed several time, Bb12 can also be found in literature and in commercialised products as *B. bifidum* and *B. animalis*.

This microorganisms have been shown to exhibit inter alia the following properties: they are gram +, non motile and nonsporing rods, resistant to gastrointestinal acidity
15 and bile salts. They are catalase negative, and produce only L(+) lactic acid and not its D(-) isomer. They can utilize the following carbohydrates: ribose, saccharose, D-glucose, D-raffinose, maltose, melibiose, amygdalin and beta-gentobiose.

They can be added to any kind of infant, baby, or childhood food product, such as
20 malted milk, infant formulae, follow up formulae, baby cereals and the like. Sometimes, diversification of the infant alimentation can occur as early of four months of life, and the bifidobacteria can be incorporated to the infant food given to children aged four months and older. As milk hypersensitivity in the majority disappears at the age of 2 to 3 years, supplementation of infant and baby food with Bifidobacterium to maintain oral
25 tolerance can be done in every food product for infants and babies from 4 months to 3 years. But supplementation can also be realised in food products not specifically aimed for infant or baby nutrition and nevertheless being part of their alimentation, for example milk, yoghurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, or milk based products, among others.

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The amount of microorganism added to the infant food product, baby food product or food product in general should be of from 10^5 to 10^8 cfu/mL of reconstituted formula or cfu/g of food product, preferably 10^5 to 10^7 , and in a most preferred embodiment 10^6 . However these amounts should not be considered as limitative and should be adapted to
35 the aimed population, for example based on the weight and age of the infant or baby, or specific populations such as population having specific diseases, for example infants, babies or children having gut diseases, acute diarrheas or inflammatory syndroms, among others.

The aforementioned Bifidobacteria, being in particular a *Bifidobacterium lactis* and in a preferred embodiment Bb12 *Bifidobacterium lactis* ATCC 27536 can also be incorporated in pet food products in order to maintain oral tolerance. It can be incorporated in chunk products or croquettes preferably, but it can also be incorporated in humid products, such as cans, for example. It can also be added to liquid formulas for pets, such as milk for kittens or puppies, among others, and can as well be added to chew products for pets.

The supplementation of pet food by this micro-organism can be comprised, for example, between 10^4 and 10^8 cfu/g for non humid products, 10^5 to 10^8 cfu/g for humid products, and 10^5 to 10^8 cfu/g for liquid products, these amounts not being limitative.

We have shown that a cell-free extract of *B. lactis* Bb12 hydrolyzes tryptic-chymotryptic peptides of BLG and thereby both decreases their IgE recognition and increases their *in vitro* stimulation of Th1 cells. Acidic TC peptides are better splenocyte proliferation inducers than basic peptides. However, when further hydrolyzed by *B. lactis* cell-free extract, acidic and basic peptides exhibit similar stimulating effects. This stimulating effect is more pronounced in naïve mice than in BLG tolerant mice.

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The relative resistance of proteins such as BLG to proteases allows portions of them to remain intact after digestion, making them potentially highly allergenic. It is generally accepted that hydrolysis of proteins by trypsin and/or chymotrypsin decreases their allergenicity. Consistent with this finding, we observed that the IgE binding capacity of tryptic-chymotryptic peptides of BLG is lower than for native BLG.

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Although not wishing to be bound by theory, we believe that the mechanism of intervention by these potential anti-allergy strains appears to be regulation of the pro-Th1/anti-Th2 response through the enhancement of IFN- γ production, a Th1 cell-related cytokine. IFN- γ is a potent immunoregulatory and inflammatory cytokine which has been found at low levels in neonates and deficient in patients with food allergy. IFN- γ plays a crucial role in food allergy prevention by inhibiting Th2 differentiation decreasing levels of IL-4 and hence inhibition of IgE production. We observed that cell free extract alone from *B. lactis* Bb12 induces IFN- γ production by splenocytes. These findings strongly suggest that bacteria such as *B. lactis* Bb12 enhance the Th1 profile

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either directly or via degradation of potentially allergenic proteins after cell lysis and liberation of cytoplasmic contents into the intestinal tract.

In addition to the previously mentioned effects of IFN- γ , it has been reported that IFN- γ may increase absorption of intact antigens across the gut mucosa, suggesting a potential unfavorable effect of *B. lactis* Bb12 for subjects with hyperresponsiveness to milk proteins. However, antigen transport across Peyer's patches has also been enhanced by IFN- γ . Since Peyer's patches are the site of secretory IgA formation following oral antigen exposure, local stimulation of IFN- γ production by *B. lactis* Bb12 may inhibit penetration of potentially harmful dietary antigens via the mechanism of immune exclusion. In agreement with this finding, increased fecal IgA levels have been observed in infants fed *B. lactis* Bb12-containing follow-up formula.

Our results show that *B. lactis* Bb12 exhibits anti-allergy properties through a mechanism of immune deviation enhancing a pro-Th1/anti-Th2 cell response and through mechanisms of hydrolysis and immune exclusion of potentially harmful dietary antigens. We investigated whether Bb12 can induce IFN- γ production in young mice never previously exposed to BLG ('naïve' mice). Production of IFN- γ in response to *B. lactis*-degraded fractions is more apparent with splenocytes of naïve mice than of mice rendered tolerant by feeding a BLG-enriched diet, suggesting that *B. lactis* Bb12 maintains the Th1 response in bottle-fed neonates and in older babies, counterbalancing the Th2-polarized immunity of newborns.

Preferably, a combination of a Lactobacillus and a Bifidobacteria to both promote and maintain oral tolerance, in particular to cow's milk will be used. In a preferred embodiment, the food product could comprise *Lactobacillus paracasei* and *Bifidobacterium lactis* to achieve the desired purpose. For example, the combination could comprise *Lactobacillus paracasei* CNCM I-2116 and *Bifidobacterium lactis* ATCC 27536. The means to promote and maintain oral tolerance can be a combination of one or several Lactobacilli with one or several Bifidobacteria. For example, it can be a combination of *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis*. Depending on which population will ingerate the food supplemented with the microorganisms, the proportion of Lactobacilli compared to Bifidobacteria might vary. For example, for a population of infants which have not yet

been in contact with cow's milk, the Lactobacilli:Bifidobacteria ratio could be comprised between 10:1 and 1:1. For a population of infants, babies or children having previously ingested cow's milk (or any other allergen or antigen), the Lactobacilli:Bifidobacteria ratio could be comprised between 1:10 and 1:1.

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If more than one Bifidobacterium is incorporated to the food product, the ratio of one Bifidobacteria compared to the other can be from 0.01 to 99.9%, and the same applies if there is more than one Lactobacillus in the preparation.

- 10 The food product in which the combination of micro-organisms according to the invention is added can be any food product listed in the description of the first aspect of the invention and the second aspect of the invention, as well as any other food product, for example chocolate, chocolate powder, spreads, pastries, jellies, jams, biscuits, snacks, juices, dairy products, breakfast cereals, and more generally any food product
- 15 eaten by infants, babies or children, as well as any food product eaten by pets.

- The amount of microorganisms in the foodstuff according to the invention is preferably comprised between 10^4 and 10^8 cfu/g of food product or cfu/mL of reconstituted product when incorporated to humid or liquid products, and 10^4 to 10^9 cfu/g of food
- 20 product when incorporated to non-humid or less humid products. The ratio Lactobacilli:Bifidobacteria does not interfere on the quantity of microorganisms added into the food product.

- Particularly, microorganisms of the invention, alone or in combination, help to prevent
- 25 and maintain tolerance of ingested antigens. It is particularly suitable for food allergies, such as allergies to shellfish comprising shrimp, crayfish, lobster and crab, to peanuts, eggs, tree nuts (for example walnuts or cashew), soy, wheat, fish, and any other known food allergy. It is also suitable for other allergies, such as allergies to acarids, pollens or dusts, among others.

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- Probiotics may also be used in the manufacture of a food product to prevent from the risk of rejecting transplants. Such food product can comprise at least one member of the lactic acid bacteria family to induce oral tolerance, and/or at least one member of the Bifidobacteria genus to maintain an oral tolerance previously induced. The lactic acid
- 35 bacteria strains belonging to the Lactobacillus genus are preferred, in particular *Lactobacillus paracasei*, and more particularly *Lactobacillus paracasei* CNCM I-2116. The Bifidobacteria used is preferably a *Bifidobacterium lactis*, and more particularly the Bb 12 strain ATCC 27536.

Indeed, although not wishing to be bound by theory, we believe that these two bacteria have the ability to specifically stimulate the immune system by promoting oral tolerance. Consequently, the mechanism developed by the immune system together with these bacteria, alone or in combination, moving away allergies is not specific to
5 cow's milk allergy or hypersensitivity, and this mechanism can help patients in need of a transplantation or having been transplanted not to reject their transplant. An aspect of the present invention is so to prevent the risk of graft versus host diseases appearance by helping to the induction and the maintain of the oral tolerance to the graft, prior the transplantation and thereafter, in order to help patients to support transplantation, from
10 an immunological point of view, by acquiring oral tolerance to the antigen that will be transplanted..

The microorganisms added to the food products according to the invention are preferably alive but can also be dead or can be inactivated for example by
15 lyophilisation. Indeed, the external membrane of the dead bacteria would seem to activate the immune system, at least partly, in the same way than the alive bacteria. Consequently, in any embodiment of the invention, the bacteria or combination of bacteria can be achieved with dead organisms. In this case, the amount of microorganisms incorporated to the food product is preferably enhanced compared to
20 the amounts given above, from 5% to 250%.

Examples

The following examples are illustrative of some of the products and methods of making
25 the same falling within the scope of the present invention. They are not to be considered in any way limitative of the invention. Changes and modifications can be made with respect to the invention. That is, the skilled person will recognise many variations in these examples to cover a wide range of formulas, ingredients, processing, and mixtures to rationally adjust the naturally occurring levels of the compounds of the invention for
30 a variety of applications.

Example 1: induction of oral tolerance

3 groups of 20 germ-free female mice C3H/HeJ 3 to 5 weeks old and one group of
35 conventional female mice BALB/c 3 weeks old were used in this experiment. Two probiotic strains were used in the study: one isolated from faeces of healthy babies was

from the Nestlé Culture Collection (Lausanne, Switzerland): *Lactobacillus paracasei* NCC 2461 (CNCM I-2116) and one was purchased from Chris Hansen (France) and is of human origin: *Bifidobacterium lactis* Bb12 NCC 362.

The germ free mice were inoculated by the oral route with an intragastric tube with 0.3 mL of a 24 hours bacterial culture containing around $5 \cdot 10^8$ cfu/mL of one out of the *L. paracasei* or *B. lactis* strains. Oral induction was induced two weeks after bacterial feeding. In each group, 10 mice were given oral administration of 3 mg/g body weight whey proteins to induce oral tolerance. Whey proteins were obtained by ultrafiltration of acid whey, and the protein content was 80%, approximately 62% of which was β -lactoglobulin. The remaining 10 mice orally received a single feeding of saline water as negative control (non-tolerized mice).

Five days later, all mice were immunised subcutaneously with 100 μ m β -lactoglobulin (BLG) 3 times crystallised and 100 μ g ovalbumin grade V (OVA). Mice were sacrificed 28 or 50 days after gavage to assess induction and maintenance of tolerance, respectively. At each sacrifice, 5 whey-protein fed and 5 water-fed mice were killed. Mice sacrificed at 50 days received two additional subcutaneous injections of BLG and OVA at day 21 and 35.

The amount of BLG-specific IgE and BLG-specific IgG1 and IgG2a levels in serum were determined by ELISA.

The induction of oral tolerance in conventional, germfree and monoassociated mice were assessed in mice sacrificed 28 days after oral administration of whey proteins and immunised with BLG: see figure 1A. The results clearly showed that whey proteins feeding significantly suppressed ($P < 0.05$) the BLG-specific IgE, IgG1 and IgG2a antibody responses in conventional, germfree and *L. paracasei*-associated mice indicating a Th1- and Th2- type humoral responses suppression. In mice associated with *B. lactis*, only anti-BLG IgE response was significantly suppressed: anti-BLG IgG1 response was suppressed but not significantly ($P < 0.2$). Hence, only the Th2-type humoral responses were sensitive to tolerance in this group of mice.

Induction of tolerance in terms of spleen cells proliferation and cytokine production: to further confirm that tolerance induction is modulated by probiotics, spleen cells were cultured in vitro and both cell proliferation (table 1) and cytokine profiles in culture supernatants were examined (figure 2A)

Table 1 Proliferative response of BLG- or PHA-activated splenocytes from conventional (Conv), monoassociated and germfree (GF) mice killed 28 days or 50 days after oral feeding with whey proteins.

	Proliferative index									
	Proliferation (cpm)						Stimulation index			
	Day 28			Day 50			Day 28		Day 50	
	Ag(-)	BLG	PHA	Ag(-)	BLG	PHA	BLG	PHA	BLG	PHA
Conv	212	3.174	70.541	311	7.214	86.268	15	332	23	277
B. lactis	273	13.355	30.328	401	27.403	53.806	49	111	61	134
L. paracasei	361	5.881	45.341	763	20.919	78.489	16	126	27	102
GF	281	8.302	58.284	256	33.744	40.64	29	207	131	158

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Example 2: immunosuppressive effects of *Lactobacillus paracasei* NCC 2461: incidence on oral tolerance induction to β -lactoglobulin

Molecular size of native BLG and its TC fractions before and after degradation with *L. paracasei* extract.

To assess whether *L. paracasei* extract hydrolyzes BLG and its TC fractions, molecular size of peptides before and after degradation with *L. paracasei* extract was compared. Native BLG was not at all degraded by *L. paracasei* extract while both acidic and basic TC fractions were. Acidic peptides with high molecular weights (1000-5000 Da) were
 15' hugely degraded, releasing small peptides with molecular weights lower than 1000 Da. Small peptides represent more than 75% of the degraded-fraction compared to 36% before, 42% of the released peptides being lower than 500 Da. The basic fraction was degraded to a lesser extent. Peptides ranging from 500 to 1000 Da decreased in the fraction to give peptides smaller than 500 Da, the latest representing 43% of the fraction
 20 after degradation compared to 37% before. Surprisingly, peptides with molecular weights higher than 2000 Da increased in the degraded-fraction.

Effect of BLG and its TC fractions degraded or not by *L. paracasei* extract on lymphocyte proliferation.

25 Native BLG and basic TC fraction have no effect on lymphocyte proliferation even after hydrolysis with *L. paracasei* extract regardless of concentration. In contrast, acidic TC fraction stimulated lymphocyte proliferation when used at concentrations higher than

1,000 $\mu\text{g/ml}$ as shown by stimulation indices higher than 1.8 and reaching 2.5 at 2,000 $\mu\text{g/ml}$. After degradation with *L. paracasei* extract, this stimulating effect was significantly repressed ($p < 0.05$), the strongest suppression being observed at 2,000 $\mu\text{g/ml}$. In addition, the immunosuppressive effect of *L. paracasei*-degraded acidic fraction was found more apparent with splenocytes of mice never previously exposed to BLG, showing reduction of stimulation index of 0.7 compared to 0.4 for BLG-primed mice.

10 *Effect of BLG and its TC fractions degraded or not by L. paracasei extract on cytokine production.*

Low levels of IFN- γ and IL-10 were produced by splenocytes in response to native BLG and basic TC fraction degraded or not with *L. paracasei* extract. The acidic TC fraction was found to induce high level of IFN- γ and low level of IL-10 while its degradation with *L. paracasei* extract induces the opposite response. IL-4 was found to be produced at low levels regardless of fraction but degradation of BLG and acidic fraction reduced its secretion at levels close to the detection limit of the test.

Effect of L. paracasei extract on lymphocyte proliferation and cytokine production.

To better understand the mechanism by which *L. paracasei* can induce oral tolerance to BLG in mice, the effect of the cytoplasmic extract alone on lymphocyte proliferation and cytokine production has been investigated. *L. paracasei* extract suppressed the mitogen-induced lymphocyte proliferation in dose dependant manner with maximal suppression at low dilution (1/10) and no effect at high dilution (1/1000). The cell proliferation observed in absence of PHA was not affected by *L. paracasei* extract even at high dose (dilution 1/10) suggesting that the concentrations used were not toxic for cells. Moreover, a large amount of IL-10 was produced when cell proliferation was strongly repressed while production of IFN- γ was not affected and remained very low.

Example 3: maintenance of oral tolerance

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The experiment is the continuation of example 1.

The maintenance of specific antibody response suppression in tolerized mice was monitored for a 7-week period after whey proteins feeding (figure 1). BLG-specific IgE, IgG1 and IgG2a responses were maintained suppressed in conventional mice ($p < 0.01$).

The suppression of anti-BLG IgG2a titer in germ free and *L. paracasei*-associated mice was not maintained, whereas both specific IgE and IgG1 levels were. A continued suppression of BLG-specific IgE responses and a significant decrease anti-BLG IgG1 titer were observed in mice colonised with *B. lactis*. As result, Th1- and Th2- type humoral responses were maintained suppressed in conventional mice, while only Th1- type antibody titers were maintained abrogated in germfree, *B. lactis* and *L. paracasei*-associated mice.

IFN- γ productions by spleen cells in mice sacrificed 50 days after oral tolerance induction were similar to those obtained after 28 days, except for germfree and *B. lactis*-associated mice for which the decrease became not significant and significant, respectively (Figure 2B). The Th1-cell activity also remained suppressed in all groups of mice (Figure 2B). In contrast, the high production of IL-10 was maintained only in conventional mice given whey proteins (Figure 2B). Nevertheless, spleen cells from germfree and *B. lactis*-associated mice have been found to be sensitive to long-term tolerance persistence clearly shown by high stimulation indexes reaching 131, 61 and 38, respectively, in presence of BLG (Table 1). Lower stimulation indexes were obtained in conventional mice and mice colonized with *L. paracasei* in presence of BLG, while cells from all the groups of mice responded well to PHA (Table 1).

20 **Example 4: anti-allergy proeperties of Bifidobacterium lactis Bb12 against bêta-lactoglobulin.**

Characterization of BLG TC fractions - effect of *B. lactis*-driven hydrolysis on the molecular size of BLG and its TC peptides.

25 Both acidic and basic fractions were degraded by *B. lactis* extract while native BLG did not. Before *B. lactis* degradation, the acidic fraction was composed of 36% peptides with molecular weights ranging from 2000 to 5000 Da. After *B. lactis* degradation, the percentage of these peptides decreased to 21% while low molecular weight peptides (<500 Da) increased from 19% to 46%. Small peptides were also released after basic
30 fraction hydrolysis. Basic peptides with molecular weights from 500 to 2000 Da were breakdown to give small peptides with delayed retention time.

IgE binding capacity of BLG and its TC fractions

Binding capacity of BLG and its basic TC fraction is not significantly reduced by
35 degradation with *B. lactis* extract. In contrast, serum IgE binding by acidic TC peptides was slightly reduced by *B. lactis*-driven hydrolysis.

Effect of BLG and its TC fractions degraded by *B. lactis* extract on lymphocyte proliferation

Before *B. lactis* degradation, lymphocyte proliferation was significantly stimulated in the presence of acidic fraction at concentrations of 1,000 $\mu\text{g ml}^{-1}$ or more but not in the presence of BLG and basic fraction regardless of concentration. In contrast, after degradation with *B. lactis* extract, BLG and its TC fractions all stimulated lymphocyte proliferation in a dose dependent manner. BLG and basic fraction induced significant cell proliferation at concentrations equal to or higher than 1,000 $\mu\text{g ml}^{-1}$ while acidic fraction did so at a concentration of 2,000 $\mu\text{g ml}^{-1}$. Moreover, stimulation indices obtained at 2,000 $\mu\text{g ml}^{-1}$ were similar for BLG, acidic and basic fractions. Although acidic and basic TC peptides had distinct stimulating effects, degradation with *B. lactis* extract produced converging effects at high dose.

Since BLG hydrolyzed by *B. lactis* extract, unlike acidic and basic fractions, was not subsequently filtered to remove enzymes, the effect of the extract alone on lymphocyte proliferation was assessed as a control. Diluted to 1/100 (as for BLG hydrolysis, wells containing 2000 $\mu\text{g ml}^{-1}$ of BLG also containing 40 $\mu\text{g ml}^{-1}$ of cell extract protein), the extract strongly stimulated lymphocyte proliferation suggesting that the stimulating effect of BLG may have been due mainly to cell-free extract present at 40 $\mu\text{g ml}^{-1}$. In contrast, ultrafiltration of the *B. lactis* extract itself produced permeate essentially free of proteins as verified by C18 chromatography suggesting that only traces of the extract was remaining in both acidic and basic fractions after filtration. At low concentration (1/1000 or 4 $\mu\text{g ml}^{-1}$ of cell extract protein), the extract did not likely stimulate lymphocyte proliferation indicating no contribution to the observed effect of acidic and basic fractions.

Proliferation of lymphocyte from naïve and tolerant mice

This paragraph deals with effect of *B. lactis* extract on the factors used to stimulate *in vitro* proliferation of lymphocytes from young naïve mice and mice daily receiving a BLG-enriched diet. Before degradation by *B. lactis* extract, the acidic fraction stimulated lymphocytes from naïve mice more than those from their tolerant counterparts while BLG and basic peptides did not. After degradation with *B. lactis* extract, BLG and its TC fractions all stimulated significantly stronger proliferation of

lymphocytes from naïve mice compared to tolerant mice as indicated by indices reaching 4 compared to 3. However, the *B. lactis* extract alone (dilution 1/100) was shown to stimulate lymphocyte proliferation in naïve mice as previously mentioned for tolerant mice, suggesting no real stimulating effect of native BLG degraded by *B. lactis* extract on proliferation.

Cytokine production

This paragraph deals with the production of cytokines by splenocytes from naïve and tolerant mice in the presence of BLG and its TC fractions, hydrolyzed or not by *B. lactis* extract. In naïve and tolerant mouse splenocyte cultures, IFN- γ was produced at low levels in the presence of untreated BLG, acidic and basic TC fractions, with the highest production of 400 pg ml⁻¹ obtained in response to acidic fraction. When hydrolyzed by *B. lactis* extract, BLG and fractions strongly induced IFN- γ secretion by both naïve and tolerant mouse splenocytes with higher production for naïve mice. The same profile of response was observed for IL-10 secretion but the levels of production were ten times lower than for IFN- γ . No IL-4 or IL-5 was detected in cell culture supernatants regardless of BLG sample or mice, suggesting no strong Th2-type cytokine induction. As observed for lymphocyte proliferation, the production of IFN- γ in response to BLG degraded by *B. lactis* extract is due mainly to extract remaining in the sample.

Example 5: infant formulae promoting and inducing oral tolerance

We prepare an infant formula by mixing together the following ingredients in the indicated proportions. The final product comprises 519 kcal for 100 g, and is in powder form.

25	Fat	27.7 g
	Fat from milk	0.7 g
	Mixture of fats (150)	26.8 g
	Lecithin	0.2 g
30	Linoleic acid	4.1 g
	α -linolenic acid	525 mg
	Proteins	9.5 g
	Free carbohydrates	57.9 g
	Lactose	57.9 g
35	Minerals (ashes)	1.9 g

	Sodium	120 mg
	Potassium	460 mg
	Chlorure	330 mg
	Calcium	320 mg
5	Phosphorus	160 mg
	Magnesium	36 mg
	Manganese	40 µg
	Selenium	10.4 µg
	Total solids	97.0 g
10	Humidity	3.0 g

We then add to the above-prepared formulae 10^6 per 100g of *Lactobacillus paracasei*, and 10^2 of *Bifidobacterium lactis*.

15 Example 6: cat food promoting and inducing oral tolerance

A feed mixture is made up of about 58% by weight of corn, about 6% by weight of corn gluten, about 23% by weight of chicken meal, salts, vitamins and minerals making up the remainder.

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The feed mixture is fed into a preconditioner and moistened. The moistened feed is then fed into an extruder-cooker and gelatinised. The gelatinised matrix leaving the extruder is forced through a die and extruded. The extrudate is cut into pieces suitable for feeding to cats, dried at about 110°C for about 20 minutes, and cooled to form pellets. At this point, a lyophilised powder of one or more strains of the following species is provided for application to the pellets: *Lactobacillus paracasei* and *Bifidobacterium lactis*. Sufficient powder is thus provided so that the corresponding dietary intake amount for the cat is from about 10^7 - 10^9 cfu / day . Some of the powder is mixed into a first mass of pellets and bagged. A second quantity of the powder is measured out and mixed with a lipid carrier which is then sprayed on to a second mass of pellets. The pellets are bagged after the coating has dried sufficiently at 50-60°C for some minutes.

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Claims

1. Use of a *Bifidobacterium* strain for the manufacture of a food product for the maintenance of oral tolerance to antigens.
- 5 2. Use according to claim 1 wherein the *Bifidobacterium* is a *Bifidobacterium lactis*.
3. Use according to claim 1 or claim 2 wherein the *Bifidobacterium* is *Bifidobacterium lactis* ATCC 27536.
- 10 4. Use according to one of claims 1 to 3 wherein the added bacteria are inactivated or dead.
5. Use according to one of claims 1 to 4 wherein the food product is an infant formula.
- 15 6. Use according to one of claims 1 to 5 wherein the bacteria are present in the food product in an amount of 10^5 to 10^8 cfu/mL of reconstituted formula or cfu/g of food product, preferably 10^5 to 10^7 , and in a most preferred embodiment 10^6 .
- 20 7. Use according to one of claims 1 to 4 wherein the food product is a pet food.
8. Use according to claim 7 wherein the bacteria are present in an amount of 10^4 and 10^8 cfu/g for non humid products, 10^5 to 10^8 cfu/g for humid products, and 10^5 to 10^8 cfu/g for liquid products.
- 25 9. Use of a *Lactobacillus* and a *Bifidobacteria* for the manufacture of a food product for the promotion and maintenance of oral tolerance to antigens. .
- 30 10. Use according to claim 9 wherein the *Lactobacillus* is a *Lactobacillus paracasei* and the *Bifidobacterium* is a *Bifidobacterium lactis*.
- 35 11. Use according to claim 10 wherein the *Lactobacillus paracasei* is *Lactobacillus paracasei* CNCM I-2116 and the *Bifidobacterium lactis* is *Bifidobacterium lactis* ATCC 27536.

12. Use according to one of claims 9 to 11 wherein the food product is an infant formula.
13. Use according to claim 12 wherein the infant formula is administered prior to contact with an antigen and the Lactobacilli:Bifidobacteria ratio is comprised between 10:1 and 1:1.
14. Use according to claim 12 wherein the infant formula is administered after contact with an antigen and the Lactobacilli:Bifidobacteria ratio is comprised between 1:10 and 1:1.
15. Use according to one of claims 9 to 11 wherein the food product is a pet food.
16. Use according to one of claims 9 to 15 wherein the amount of microorganisms in the foodstuff according to the invention is preferably comprised between 10^4 and 10^8 cfu/g of food product or cfu/mL of reconstituted product when incorporated to humid or liquid products, and 10^4 to 10^9 cfu/g of food product when incorporated to non-humid or less humid products.
17. Use of probiotics for the manufacture of a food product to decrease the risk of rejecting transplants.
18. Use according to claim 17 comprising a Lactobacillus to induce oral tolerance, and a Bifidobacterium to maintain an oral tolerance previously induced.
19. Use according to claim 18 wherein actobacillus is *Lactobacillus paracasei*, preferably *Lactobacillus paracasei* CNCM I-2116.
20. Use according to claim 19 wherein the Bifidobacterium a *Bifidobacterium lactis*, preferably Bb 12 strain ATCC 27536.