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GRN 000531

Granada, July 16th 2014

Dear Madam/Sir,

Please find enclosed GRAS notification for the product *Lactobacillus fermentum* CECT5716 .

The intended uses of *Lactobacillus fermentum* CECT5716 are to enrich foods in these beneficial bacteria, so-called probiotics.

Three signed copies of the document containing detailed data and information that serve as the basis for this GRAS determination and an electronic copy is enclosed.

If you have any questions, please do not hesitate to contact me.

Best regards,

(b) (6)

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FOOD ADDITIVE SAFETY
GRN 000531

GRAS NOTIFICATION

Lactobacillus fermentum CECT5716

Version 1

biosearch life

Granada July 2th 2014

GRAS NOTIFICATION FOR LACTOBACILLUS FERMENTUM CECT5716

GRAS Exemption Claim

Biosearch Life hereby notifies the Food and Drug Administration that the use of *Lactobacillus fermentum* CECT5716 described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Biosearch Life has determined that such use is Generally Recognized As Safe (GRAS).

(b) (6)

July 2nd , 2014

José Mª Roset Monrós

CEO, Biosearch Life

Date

Name and Address of Notifier

Biosearch Life, S.A.
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PART A GRAS DETERMINATION

A.1 Identity of GRAS Substance

The substance that has been determined to be Generally Recognized As Safe (GRAS) and is thus the subject of this GRAS notification is *Lactobacillus fermentum CECT5716*. This bacterial strain is manufactured by Biosearch Life, and was initially isolated from human breast milk (Martín et al 2005). The strain has been deposited in the Spanish Culture Collection, hence the CECT number, and has been exhaustively characterized using microbiological, biochemical and genetic techniques (Martín et al 2003; Martín et al 2005). This substance has not been genetically modified.

A.2 Intended Use and Consumer Exposure

The intended uses of *Lactobacillus fermentum CECT5716* are to enrich foods in these beneficial bacteria, so-called probiotics. For this reason the technical effect that covers the use of this Lactobacillus strain presented herein can be defined by the purpose of providing a benefit to the consumer of the food, or, in other words, a nutrient supplement (definition #20 of the Code of Federal Regulations 21 CFR 170.3) not to be confused with a dietary supplement.

The food category to which *Lactobacillus fermentum CECT5716* is intended to be added is presented in Table I. The maximum levels are also presented in this Table for each category. The use of *Lactobacillus fermentum CECT5716* is limited to infants and young children.

A.3 Basis for the GRAS Determination

The GRAS determination for *Lactobacillus fermentum CECT5716* under the proposed uses and at the maximum use levels listed in Table I is based on scientific procedures as described under title 21 of the Code of Federal Regulations (21 CFR §170.30(b)).

To demonstrate that *Lactobacillus fermentum CECT5716* is GRAS under their intended conditions of use, the safety of the intake of this bacterial strain is established under its intended conditions of use. This intake of *Lactobacillus fermentum CECT5716* is

determined to be safe by showing that the safety of these levels of intake is generally recognized by experts qualified by scientific training and experienced to evaluate the safety of food substances, and is based on generally available and accepted information.

The publicly available data demonstrating the safety of the proposed uses of *Lactobacillus fermentum CECT5716* was reviewed by an Expert Panel consisting of:

Luis Moreno Aznar, PhD Professor University of Zaragoza, Spain
Andreu Palou, PhD Professor University of Islas Baleares, Spain
Ángel Gil Hernández, PhD Professor University of Granada, Spain

The undersigned, an independent panel of recognized experts, qualified by their scientific capacity and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Biosearch Life S.A to determine the Generally Recognized As Safe (GRAS) status of *Lactobacillus fermentum* CECT5716 to enrich foods in these beneficial bacteria, so-called probiotics (as a nutrient supplement (definition #20 of the Code of Federal Regulations 21 CFR 170.3).

A comprehensive search of the scientific literature for safety and toxicity information on *Lactobacillus fermentum* CECT5716 was conducted through March 2014 by experts of Biosearch Life S.A. and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Biosearch Life S.A and other information deemed appropriate or necessary. Biosearch life accepts responsibility for the GRAS determination that has been made for *Lactobacillus fermentum* CECT5716 as described herein.
Following an independent, critical evaluation, the Expert Panel conferred on May 19th, 2014 and unanimously agreed to the decision described herein.

This Expert Panel concluded that:

Lactobacillus fermentum CECT5716 has been sufficiently characterized to ensure that it is a food grade product, and, therefore, Lactobacillus fermentum CECT5716, meeting the food grade specifications described in this notification, is safe and GRAS under their intended conditions of use.

In the opinion of these experts, other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion. It is therefore concluded that, based on scientific procedures, the intended use of *Lactobacillus fermentum CECT5716*, as shown in Table I, is safe and GRAS. Because *Lactobacillus fermentum CECT5716* is GRAS for their proposed uses, it is excluded from the definition of a food additive, and thus may be lawfully marketed and sold for its use in the U.S. without the promulgation of a food additive regulation under 21 CFR.

Table I Intended food category for supplementation with *Lactobacillus fermentum CECT5716*, and their maximum addition levels. Food category was selected from the 43 food categories as defined in the Code of Federal Regulations 21 CFR 170.3.

Food Category	**Max. Levels of *L.fermentum CECT5716***
Milk products (31), particularly milk powders	1E7 cfu/gram

A.4 Availability of Information

The detailed data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request and are available for the FDA´s review and copying at Biosearch Life, contact person: Mónica Olivares, Camino de Purchil 66, 18004 Granada, Spain, telephone: +34-958240152, fax: +34-958240160, and email: molivares@biosearchlife.com.

PART B CHEMICAL AND TECHNOLOGICAL DATA

B.1 Identity of Substance

Lactobacillus fermentum CECT5716 is a proprietary strain of Biosearch Life that was isolated in research collaboration with the Department of Nutrition and Bromatology of the University Complutense of Madrid (Universidad Complutense de Madrid).

The taxonomical classification of this strain was based on the analysis of the SDS-PAGE 1D protein profiling and their 16S rDNA sequence, and in order to discriminate between this strain and other Lactobacillus species, RAPD-PCR analysis was performed using two specific primers for lactobacilli (ArgDei and OPL5) (Martín et al 2003).

Besides the genetic patterns, the strain was characterized by its carbohydrate metabolic pattern (API 50; BioMerieux), enzymatic activities (APIZYM; BioMerieux) and antibiotic resistance (Martín et al 2005, Lara-Villoslada 2009).

The strain has been deposited according to the Budapest Agreement at the Spanish Culture Collection (CECT; Colección Española de Cultivos Tipo) with the following accession number:

Lactobacillus fermentum: CECT5716

Lactobacillus fermentum CECT5716 has not been genetically modified, and has no plasmids. The total genome of Lactobacillus fermentum CECT5716 has been sequenced and is available in GenBank/EMBL under accession no. CP002033.

B.2 Description of Manufacturing Process

Lactobacillus fermentum CECT5716 is produced as a pure culture. The manufacturing process consists of three batch fermentation steps followed by the concentration of the biomass by centrifugation, the addition of cryoprotectants, and a freeze-drying process.

The production process is performed under ISO 22000:2005 standards as certified by IQ-Net (registration number: ES-SA-0035/2011).

B.2.1. Strain Maintenance

Biosearch Life maintains a cell bank system of all its strains. This system is based on a Master Cell Bank (MCB) of each strain and Working Cell Banks (WCB) that is prepared when needed, starting from a vial of the Master Cell Bank. The Master Cell Bank consists of several frozen vials stored at -80ºC.

Each time a WCB has to be prepared, a vial of the MCB is plated and one isolated colony is grown in a selective culture media. After 16h of growth, the broth is centrifuged and the biomass is resuspended in the same volume of media plus 10% sucrose. This cell suspension is divided into portions and store at -80ºC.

Before the new WCB is liberated to be used as starting material for production, it is checked in terms of potency, purity and identity.

Identity is confirmed by:

- Colony appearance and cell morphology under microscopic observation.
- Carbohydrated metabolic pattern
- qPCR with strain-specific oligonucleotides and probes for each strain

B.2.2. Fermentation

Lactobacillus fermentum CECT5716 is grown by standard fermentation techniques and under conditions that are suitable for human food use. All raw materials used meet specifications of the Food Chemical Codex. The culture media consist of a sugar as carbon and energy source, a complex nitrogen source (yeast extract), vitamins and minerals.

In order to grow the selected strain as a pure culture, all required measurements well known in fermentation technology are applied to avoid contamination by foreign microorganisms present in the environment.

B.2.3. Concentration and Freeze-drying

When the main fermentation is finished, cells are cooled and harvested. The biomass is concentrated by centrifugation and sent to a tank where a solution of cryoprotectants was prepared previously. The cryoprotectant solution consists of maltodextrine and sodium ascorbate.

Cells are mixed with the cryoprotectants and then frozen as pellets in liquid nitrogen. Frozen pellets are recovered in an ultra-clean area, and stored in sterile bags at -60ºC to -80ºC until freeze-drying. For freeze-drying the frozen pellets are placed in an industrial freeze-dyer for 2 to 3 days. Then, the material is harvested in an ultra-clean room, milled, and filled in sterile bags.

B.2.4. Packaging of bulk product and final product

Freeze-dried material, the bulk product, is stored at -80ºC. A sample is analyzed to determine potency, identity checked by qPCR, and the absence of microbial contamination is confirmed.

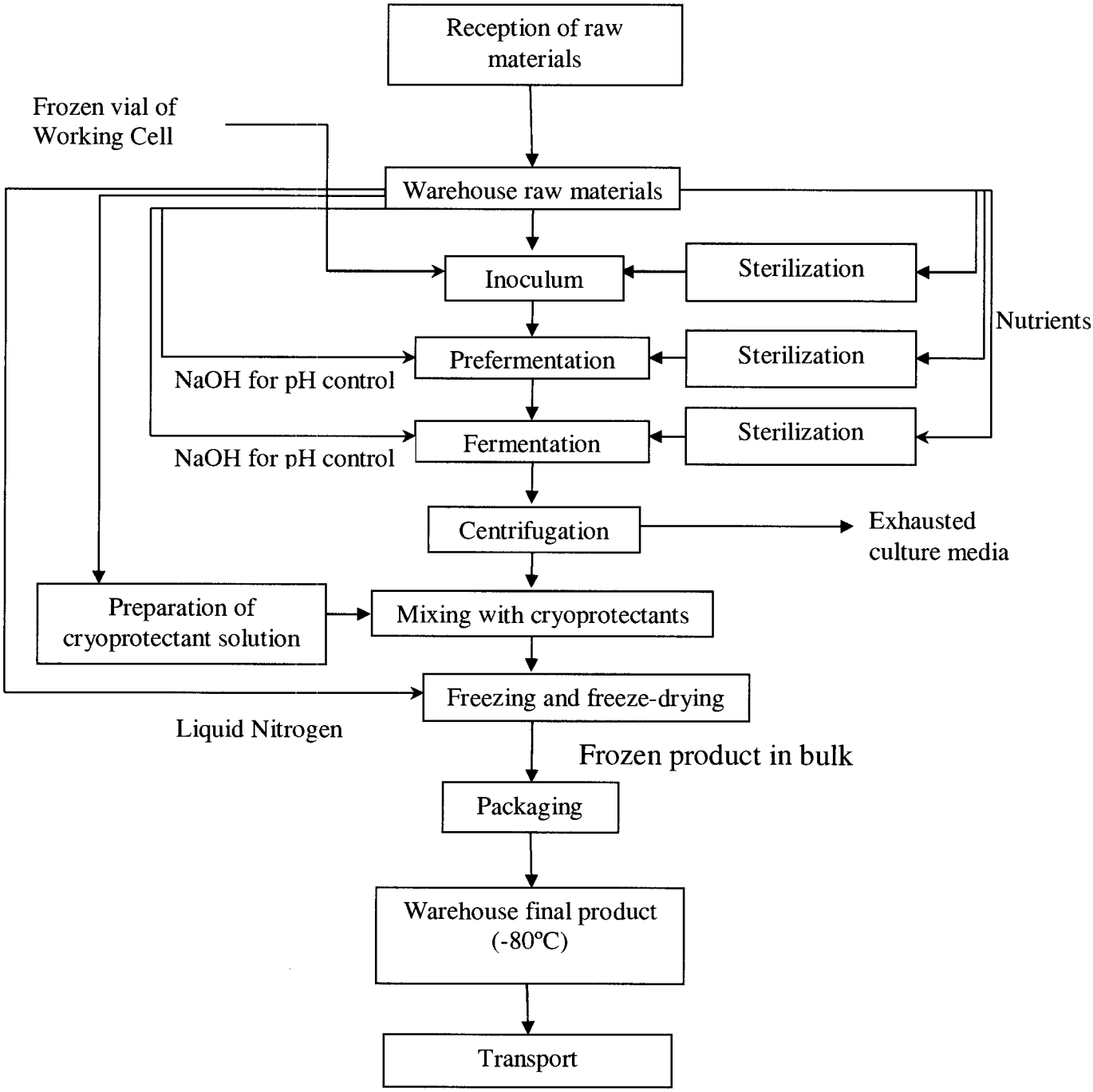
Once the bulk product is liberated by Quality Assurance, the product is packed in aluminum laminated sachets at pre-defined potency per sachet.

Representative samples are taken during packaging to confirm potency and absence of microbial contamination.

B.2.5 Storage and transport

The aluminum-laminated sachets containing the product are stored at -80ºC until delivery. The bags are transported in special boxes containing frozen elements to maintain a cool ambient.

Scheme I Schematic representation of the manufacturing process of *Lactobacillus fermentum CECT5716.*



B.3 Specifications for Food grade Material

Biosearch Life has developed specifications for *Lactobacillus fermentum CECT5716* to demonstrate that this product is food grade. These specifications are listed below, together with the standard operating procedures used by Biosearch Life for determining compliance with each specification. Each batch is checked for identity by qPCR using strain-specific oligonucleotides and probes in order to confirm the identity of the strain and the absence of other Lactobacillus strains.

• Description:	Food grade *Lactobacillus fermentum CECT5716* is produced as a freeze-dried powder, and packed in aluminium bags. Recommended storage is -20ºC (-4ºF) or lower.
• Appearance:	White-yellowish powder
• *Lactobacillus fermentum*	NLT 1E11 cfu per gram
• Yeast & Moulds	NMT 50 cfu per 1 gram
• Enterobacteria	NMT 10 cfu per 1 gram
• *Bacillus cereus*	NMT 500 cfu per 1 gram
• *Staphylococcus aureus*	NMT 10 cfu per 1 gram
• *Salmonella spp.*	absent in 25 grams
• *Lysteria monocytogenes*	absent in 25 grams
• *Escherichia coli*	absent in 1 gram
• *Enterobacter sakazakii*	absent in 10 grams
• Moisture	NMT 10%
• Water activity	NMT 0,25

B.4 Batch Analysis Results

To demonstrate conformance with the proposed specifications listed above, the analytical results of five different lots of *Lactobacillus fermentum CECT5716* are presented in Table II and III.

Table II Analytical results of five different non consecutive lots of *Lactobacillus fermentum CECT5716*

Parameter	Batch number				
	HF058	**HF075**	**HF105**	**HF133**	**HF152**
Appearance	Conform	Conform	Conform	Conform	Conform
Identity by qPCR	Conform	Conform	Conform	Conform	Conform
L. fermentum (cfu/g)	1,1E11	1,5E11	1,0E11	1,2E11	1,2E11
Yeast & Moulds (cfu/g)	n.d.	n.d.	n.d.	n.d.	n.d.
Enterobacteria (cfu/g)	n.d.	n.d.	n.d.	n.d.	<10
Bacillus cereus (cfu/g)	<10	<10	<10	2,8E1	n.d.
Staphylococcus aureus (cfu/g)	n.d.	n.d.	n.d.	n.d.	<10
Salmonella spp. (cfu/ 25g)	n.d.	n.d.	n.d.	n.d.	n.d.
Lysteria monocytogenes (cfu/ 25g)	n.d.	n.d.	n.d.	n.d.	n.d.
Escherichia coli (cfu/ 10g)	n.d.	n.d.	n.d.	n.d.	n.d.
Enterobacter sakazakii (cfu/ 10g)	n.d.	n.d.	n.d.	n.d.	n.d.
Moisture	4,8	4,6	4,1	4,3	3,3
Water activity	0,229	0,206	0,163	0,141	0,118

B5. Analytical methods

The analytical procedures used to confirm the quality of every batch are based on the methods presented in table III.

Table III **Analytical methods used to analyze each batch of *Lactobacillus fermentum CECT5716.***

Parameter	Analytical Method
Appearance	In house method
Identity by qPCR	In house method
L. fermentum (cfu/g)	ISO 15214 (1998) Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of mesophilic lactic acid bacteria - Colony-count technique at 30 ºC.
Yeast & Moulds	ISO 6611 IDF 94 (2004) Milk and milk products - Enumeration of colony forming units of yeasts and/or moulds - Colony count technique at 25 ºC.
Enterobacteria	ISO 21528-1 (2004) Microbiology of food and animal feeding stuffs - Horizontal methods for the detection and enumeration of Enterobacteriaceae - Part 1: Detection and enumeration by MPN technique with pre-enrichment.
B.cereus	ISO 7932:2004 Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of presumptive Bacillus cereus -- Colony-count technique at 30 degrees C.
S. aureus	ISO 6888-1:1999 / Amd 1:2003 Microbiology of food and animal feeding suffs – Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species). Part 1: Technique using Baird-Parker agar medium
Salmonella spp.	ISO 6579 (2003) Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp.
L. monocytogenes	ISO 11290-1:2004 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of Listeria monocytogenes – Part 1: Detection method
E.coli	ISO 7251:2005 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection and enumeration of presumptive Escherichia coli -- Most probable number technique
Enterobacter sakazakki	ISO/TS 22964 IDF/RM 210 (2006) Milk and milk products - Detection of *Enterobacter sakazakii.*
Moisture	In house method
Water activity	ISO 21807:2004Microbiology of food and animal feeding stuffs -- Determination of water activity

B6. Use in different food categories

The food category to which *Lactobacillus fermentum* CECT5716 is intended to be added is milk powder, in concrete, milk powders or infant formulas designed for infants from the age of 1 month (see also Table I). Because this bacterial strain consist of live bacteria, no heat treatments or other stress conditions can be applied during food processing once they have been added to the food matrix. For that reason, *Lactobacillus fermentum* CECT5716 is added to the formula by dry mixing, by addition of this ingredient to the powdered milk and mixed under gentle stirring.

The concentration of *Lactobacillus fermentum* CECT5716 in the finished product should be calculated according to a daily intake of 2x1E8 cfu. For a typical infant formula this means that the dosification is 1E7 cfu per gram of infant formula powder. During the shelf life of such an infant formula the viability of *Lactobacillus fermentum* CECT5716 may drop by approximately 1 log during 18-24 months.

PART C SAFETY EVALUATION

C.1 Scientific rationale for the use of *Lactobacillus fermentum.*

Probiotic bacteria are commonly defined as viable microorganisms that, when ingested in adequate amounts, exhibit a benefit for the host health. An important aspect of probiotic strains is their capabilities to colonize the human gut. *Lactobacillus fermentum* CECT5716 shows a high rate of adhesion to intestinal cells and is able to survive human gut transit (Martín et al 2005). *Lactobacillus fermentum* CECT5716 also produces compounds that help to maintain the integrity of the human gut epithelium. This strain induces the production of mucins that may protect the intestinal epithelium from physical, chemical and bacteriological damage (Olivares et al 2006). The consumption of *Lactobacillus fermentum* CECT5716 increases the production of short chain fatty acids (SCFAs) that constitute an important energy source for intestinal cells (Perán et al 2006). *Lactobacillus fermentum* CECT5716 is endowed of an anti-infectious character since it produces antimicrobial substances, such as lactic acid, which inhibit the growth of pathogenic bacteria such as *E. coli*, *Salmonella* spp., *Listeria* spp., *Staphylococcus aureus*, etc, and inhibit pathogens' adhesion to the gut mucosa favoring the elimination of pathogens with the fecal bulk. The anti-infectious activity has been revealed by the capability of *Lactobacillus fermentum* CECT5716 to protect against gastrointestinal infections caused by *Salmonella choleraesuis* in an *in vivo* murine infection model (Olivares et al 206).

In addition to their probiotic effect on intestinal function, *Lactobacillus fermentum* CECT5716 also possesses an immunoregulatory activity (Díaz-Ropero et al 2007). The consumption of this strain boosts the natural and adaptive immunological responses in healthy adults (Olivares et al 2007). Therefore, *Lactobacillus fermentum* CECT5716 is a *Lactobacillus* strain with a potent probiotic character that makes its use interesting for human nutrition.

C.2 Safety aspects

Although it has been reported that lactobacilli and bifidobacteria may invade the host body by bacterial translocation and other routes (Berg et al 1992), it seems probable that for these bacteria to cause infection both bacterial and host factors have to be involved. In fact, the isolation of probiotic bacteria from infections is likely to be the result of opportunistic infections. All cases of probiotic sepsis have occurred in immune compromised patients, with a chronic disease or debilitation. There is no report on probiotic sepsis in healthy individuals. In a review by Boyle et al. (2006) the known risks of probiotic treatments are revised. These authors reported 12 cases of bacterial sepsis temporally related to probiotic use in humans, nine cases of bacteremia, two cases of endocarditis and one case of liver abscess. All of them were associated with different risk factors such as diabetes mellitus, short gut syndrome, central nervous catheter and antibiotic diarrhea among others. *Lactobacillus rhamnosus* LGG and *Bacillus subtilis* were the most frequent isolated strains, which might be due, at least in the case of LGG, to the widespread use of these strains. However, it has been reported that, in spite of the marked increase in the use of the probiotic *L.rhamnosus* GG in Finland since 1990, no significant increase in *Lactobacillus* bacteremia attributable to probiotic strains has been observed in southern Finland (Saxelin et al 1996). Although *L. fermentum* has a long history of use in traditional foods, only one report has been found in the literature about adverse effects or infections caused by this specie of microorganism and occurred in a patient with a chronic disease and debilitation (Chery et al 2013). The infection was a documented cholecystitis which occurred in an 87 years old obese man, with medical history of diabetes mellitus, coronary artery disease, hypertension and dislipemia.

In the selection of new probiotic strains safety criteria are applied such as a long history of use in humans and the absence of pathogenic mechanisms (Collins et al. 1998). Thus, safety assessments are recommended for probiotic strains that are aimed to be incorporated into food products (Conway 1996). These safety criteria have been applied for *Lactobacillus fermentum* CECT5716.

C.3 Origin of *Lactobacillus fermentum* CECT5716

Recently it has been showed that breast milk is an important source of lactic acid bacteria for infants (Martín et al 2003). It is estimated that breast milk contains about 10^3

colony forming units of commensal bacteria per milliliter. *Lactobacillus fermentum CECT5716* was originally isolated from human milk of healthy mothers (Martín et al. 2005b).

C.4 Identification of *Lactobacillus fermentum* CECT5716

The taxonomic classification of probiotic bacteria must be accurate since this aspect can involve connotations of safety and regulation (O´Brien et al 1999). The joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) expert Consultation on Health and Nutritional properties of powder milk with live lactic acid bacteria recommended that strains should be named according to the International Code of Nomenclature, be deposited in an internationally recognized culture collection, and that strain identification be performed by phenotypic tests followed by genetic identification with methods such as DNA/RNA hybridization and 16sRNA sequencing. *Lactobacillus fermentum* CECT5716 was identified by 16S rRNA sequencing and species-specific polymerase chain reaction (PCR) (Martín et al 2003; Martín et al 2005. This strain was deposited in the Spanish culture collection (CECT) with the code CECT5716. The strain has not been genetically modified thus is not subject to the special regulation of GMO.

Furthermore, the complete genome of *Lactobacillus fermentum* CECT5716 was sequenced recently (Jimenez et al, 2010). The complete genome of Lactobacillus fermentum CECT5716 consists of a circular chromosome of 2,100,449 bp, with a GC content of 51.49%, and has no plasmids. The complete sequence is available in GenBank/EMBL under accession no. CP002033.

C.5 Metabolic activities

Probiotic bacteria could convert food components or biological secretions into secondary substances potentially harmful for the host. Production of amines during digestion of food proteins by Lactobacilli and Bifidobacteria has been proposed as a test to assess detrimental effects of probiotics (Ishibashi and Yamazaki 2001). The production of biogenic amines was evaluated by incubating *Lactobacillus fermentum* CECT5716 in the presence of amino acids in decarboxylase broth by the method of Bover-Cid and

Holzapfel (Bover-Cid and Holzapfel 1999). The strains tested did not produce biogenic amines, suggesting a low deaminase activity (Martín et al 2005). Also it is recommended not to use strains with beta-glucuronidase activity since this activity could deconjugate toxic substances bound to glucuronic acid releasing toxins that could be absorbed. *Lactobacillus fermentum* CECT5716 is not endowed with such an enzymatic activity (Martín et al 2005). It has been suggested that the ability of some bacteria to degrade mucins may facilitate the translocation of normal intestinal bacteria into extraintestinal tissues. *Lactobacillus fermentum* CECT5716 is not able to degrade gastric mucins *in vitro* (Martín et al 2005).

C.6 Determination of antibiotic resistance patterns

The emergence and the spread or resistance to antimicrobials in bacteria pose a threat to human and animal health and present a major financial cost. As with any other bacteria, antibiotic resistance exists among some lactic acid bacteria, including probiotic microorganisms (Salminen et al 1998). The joint FAO/WHO expert Consultation on Health and Nutritional properties of powder milk with live lactic acid bacteria suggested in 2001 that further research must be done related to the antibiotic resistance of lactobacilli and bifidobacteria (FAO/WHO 2001). The Consultation recommended that probiotic bacteria should not harbor transmissible drug resistance genes encoding resistance to clinically used drugs. FAO/WHO convened in 2002 a Working Group to generate guidelines and recommend criteria and methodology for the evaluation of probiotics in foods. The Working Group recommended the determination of antibiotic resistance patterns of the probiotic bacteria strains as a requirement for proving the safety of these bacteria (FAO/WHO 2002). A list of antibiotics was proposed for which the minimal inhibitory concentration (MIC) should be tested, and also MIC breakpoints *for Enterococcus faecium, E. faecalis, Pediococcus, Lactobacillus* and *Bacillus* categorizing a bacterial strain as resistant to an antibiotic. These criteria have been applied to *Lactobacillus fermentum* CECT5716 in order to discard the presence of non desirable resistances. Minimum inhibitory concentrations (MICs) to 18 antimicrobial agents were calculated. The antimicrobial agents tested included amoxycillin/clavulanic acid, ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fosfomycin, gentamycin, imipenem, linezolid, mupirocin, oxacillin, penicillin, quinupristin/dalfopristin,

rifampicin, teicoplanin, tetracycline, trimethoprim–sulfamethoxazole and vancomycin. The values of MICs for the tested antibiotics are below those breakpoints proposed (Lara-Villoslada et al., 2009).

C.7 Animal studies

Toxicity studies in animals have been considered unnecessary for lactobacilli species which have a long history of use and lacking pathogenic mechanisms. However, such studies were carried out for *Lactobacillus fermentum* CECT5716 with the aim to reinforce the data of safety of the strain. Although extrapolation of results obtained from animal studies to human has limited validity, toxicity studies in experimental animals are generally accepted as a reliable tool to assess acute toxicity. These studies, conducted using the same procedures used for testing toxicity of chemicals, have also been performed for the evaluation of probiotic safety and tolerance of *Lactobacillus fermentum* CECT5716 (Lara-Villoslada et al., 2009). This strain was daily administered for 1 month with doses of 10^{10} CFU mouse/day, doses about 10 thousand times higher than normally consumed by humans when body weight is taken into account. Results showed that oral administration of *Lactobacillus fermentum* CECT5716 to mice had no adverse effects on body weight or food intake. No bacteraemia was present in liver or spleen and there was no treatment-associated bacterial translocation into these tissues. Liver glutathione content, as well as plasma malondialdehyde concentration, was not statistically different in probiotic-treated mice when compared to control mice. Probiotic treatment did not cause changes in the biochemical and hematological parameters analyzed. Therefore the acute toxicity study demonstrated the safety of *Lactobacillus fermentum* CECT5716 under these conditions (Lara-Villoslada et al, 2009).

C.8 Human studies

Three human studies have been performed in which *Lactobacillus fermentum* CECT5716 was orally administered to adults or infants. In these studies no adverse effects related to the strains were reported. Below a summary is given of these 3 clinical studies.

1. *Clinical study in healthy adults using Lactobacillus fermentum CECT5716 (Olivares et al., 2007)*

OBJECTIVE: To evaluate the ability of the probiotic strain *L. fermentum* CECT5716 to enhance the immune response against the influenza vaccine.

STUDY DESIGN: A randomized, double blind, placebo-controlled human clinical trial was performed to address the immunological effects of an intramuscular anti-influenza vaccine. Half of the volunteers received an oral daily dose of methylcellulose (placebo) or probiotic bacteria two weeks prior vaccination and two weeks after vaccination.

DOSE: 1E10 cfu/ day of *Lactobacillus fermentum* CECT5716.

STUDY DURATION: 1 month of treatment (+ 5 months follow up).

ADMINISTRATION: Capsules.

NUMBER AND CHARACTERISTICS OF THE VOLUNTEERS: 50 healthy adults, age 22-56 (average 33 years).

RESULTS: No adverse effects associated with the intake of the probiotic strain were observed during the treatment or follow-up periods. The results showed, two weeks after vaccination, an increase in NK cells proportion in the group consuming probiotic, but not in the placebo group. The vaccination induced an increase in Th1 cytokine concentrations, and in T helper and T cytotoxic proportions in both groups; however, the group consuming probiotic bacteria showed a significant higher induction in some of these parameters. Regarding the humoral effects, induction of antibody response in placebo group could not be detected. In the case of the probiotic group a significant increase in anti-influenza specific IgAs was detected. Although an increase in total IgM was observed, changes in anti-influenza specific IgMs were not observed. Finally, the incidence of influenza like illness during 5 months after vaccination (October-February) was lower in the group consuming *Lactobacillus fermentum* CECT5716.

CONCLUSIONS: Daily administration of *Lactobacillus fermentum* CECT5716 during 1 month is safe and well tolerated in adults. Furthermore, the oral administration of the strain L potentates the immunological response of an anti-influenza vaccine and may

provide enhanced systemic protection from infection by increasing Th1 response and virus neutralizing antibodies.

2. *Clinical study in infants from 1 to 6 months of age using Lactobacillus fermentum* CECT5716 *(Gil-Campos et al., 2012)*

OBJECTIVE: To evaluate in neonates from 1 to 6 months of life, the safety and tolerance of the consumption of an initial infant formula supplemented with *Lactobacillus fermentum* CECT5716, a probiotic strain originally isolated from breast milk.

METHODS: A randomized double blinded controlled study including healthy infants was conducted in 121 infants. One month aged infants received for 5 months an infant formula supplemented with *Lactobacillus fermentum* CECT5716 (experimental group) or the same formula without the probiotic strain (Control group). The primary outcome of the study was the average daily weight gain between baseline and 4 months of life. Secondary outcomes were other anthropometric data (length and head circumference), formula consumption, and tolerance. Incidence of infections was also recorded by pediatricians.

DOSE: Infant formula of the experimental group contained 1E7 cfu of *Lactobacillus fermentum* CECT5716 per gram of milk powder.

RESULTS: No significant differences in weight gain were observed between groups at 4 months of age (29.0±7.8 *vs.* 28.9±5.7 g/day) nor at 6 months (25.1±6.1 *vs.* 24.7±5.2 g/day). There were no statistically significant differences in the consumption of the formulas or symptoms related to the tolerance of the formula. Finally, the incidence rate of diarrhea in infants of control group was 3 times higher than in the probiotic group (p=0.018).

CONCLUSION: The consumption from 1 to 6 months of life of an infant formula enriched with the probiotic strain *Lactobacillus fermentum* CECT5716 is well tolerated and safe. Furthermore, the consumption of this formula improved the health of the infants reducing the incidence of gastrointestinal infections.

3. *Clinical study in infants from 6 to 12 months of age using Lactobacillus fermentum* CECT5716 *(Maldonado et al., 2012)*

OBJECTIVES: To examine the effects of a follow-on formula containing Lactobacillus fermentum CECT5716 on the incidence of infections in infants between the ages of 6 to 12 months.

METHODS: A randomized double blinded controlled study including 188 infants at the age of 6-months was conducted. Infants were assigned randomly to either follow-on formula supplemented with *Lactobacillus fermentum* CECT5716 (experimental group, EG), or the same formula supplemented without *Lactobacillus fermentum* CECT5716 (control group, CG). The main outcome was the incidence of infections for the 6-months duration of the study.

DOSE: Infant formula of the experimental group contained 1E7 cfu of *Lactobacillus fermentum* CECT5716 per gram of milk powder.

RESULTS: There were no reports regarding adverse effects associated to the consumption of the infant formula enriched with *Lactobacillus fermentum* CECT5716. The results showed that the evolution of weight, length and head circumference was similar in both groups and the values obtained were within the range of normality.
The EG showed a significant 46% reduction on the incidence rate (IR) of gastrointestinal infections, (EG: 0.196±0.51, CG: 0.363±0.53, IR ratio=0.54, 95% CI: 0.307-0.950, P=0.032), 27% reduction on the incidence of upper respiratory tract infections (EG: 0.969±0.96, CG: 1.330±1.23, IR ratio=0.729, 95% CI: 0.46-1.38, P=0.026), and 30% reduction in the total number of infections (EG: 1.464±1.15, CG: 2.077±1.59, IR rate ratio=0.70, 95% CI: 0.46-1.38, P=0.003), at the end of the study period compared with CG.

CONCLUSIONS: Administration of a follow-on formula with *Lactobacillus fermentum* CECT5716 is safe and well-tolerated by 6 months old infants, and may be useful for the prevention of community-acquired GI and upper respiratory infections.

C.9 Qualified Presumption of Safety

In the European Union a system was proposed for a pre-market safety assessment of selected groups of microorganisms leading to a "Qualified Presumption of Safety (QPS)". In essence a safety assessment of a defined taxonomic group (e.g. genus or group of related species) was made based on four pillars: establishing identity, body of knowledge including history of use, possible pathogenicity and end use. If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded (the qualification), the group was granted QPS status. Thereafter, any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be free from the need for further safety assessment other than satisfying any qualifications specified. The QPS status refers to the micro-organisms and is not restricted to a specific application allowing the development of novel products. Microorganisms not considered suitable for QPS would remain subject to a full safety assessment in Europe.

The EFSA Scientific Committee recommended that this QPS system for microorganisms should be introduced and implemented across EFSA as an assessment tool within the framework of the current and proposed legislation for all safety considerations of microorganisms intentionally added to the food chain, regardless of purpose (EFSA 2007). The Scientific Committee elaborated in 2007 a list of microorganisms considered suitable for QPS status (EFSA 2007). The list is annually updated and currently contains 79 species of microorganisms including 35 species of Lactobacillus and 5 of Bifidobacterium (EFSA 2012). *Lactobacillus fermentum* is included in this list as QPS microorganism, and, therefore, is considered safe.

C.10 History of Use

In different countries infant formulas containing *Lactobacillus fermentum* CECT5716 are on the market. Puleva Food, one of Spain´s largest dairy industries, launched an infant formula on the Spanish market in the year 2007 called Puleva Peques®. Puleva Food sells approximately 200 tons of this product per year, and did not receive any consumer complaints that were related to negative health effects caused by the consumption of Puleva Peques®.

In many other European countries *Lactobacillus fermentum* CECT5716 can be found in the infant formulas produced by the German company Hipp. This company is adding this probiotic strain to different formulas, from stage 1 to stage 3. Hipp sells approximately 6000 tons of formula containing *Lactobacillus fermentum* CECT5716 on a yearly basis in countries like Germany, Austria, Switzerland, UK, among others.

End of submission.

References

Berg, RD (1992). Translocation of indigenous gut flora. In: Probiotics, the scientific basis. (Fuller, R., ed.). London: Chapman and Hall. 55-85.

Boyle, R., Robins-Browne, RM., Tang MLK (2006). Probiotic use in clinical practice: what are the risks? Am J Clin Nutr; 83:1256-1264.

Bover-Cid, S., Holzapfel, W.H. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol, 53:33-41.

Chery, J., Dvoskin, D., Morato, FP., Fahoum, B. (2013) Lactobacillus fermentum, a pathogen in documented cholecystitis. Int J Surg Case Rep;4(8):662-4.

Collins, JK., Thorton, G. and Sullivan, GO. (1998) Selection of probiotic strains for human applications. Int Dairy J; 8: 487–490.

Conway, PL. (1996) Selection criteria for probiotic microorganisms. Asia Pac J Clin Nutr; 5: 10–14.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal; 587,1-16.

EFSA Scientific Opinion (2012). The maintenance of the list of QPS microorganisms internationally added to food or feed. The EFSA Journal;10(12):3020

FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, October 2001. [http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf].

FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London Ontario, Canada, April 30 and May 1, 2002
[http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf].

Gil-Campos, M., López, MA., Rodriguez, MA., Romero, J., Roncero, I., Linares, MA., Maldonado, J., López-Huertas, E., Berwind, R., Ritzenthaler, KL., Sempere, L., Geerlings, A., Maldonado-Lobón, JA., Valero, AD., Lara-Villoslada, F., Olivares, M. (2011) Safety trial of a prebiotic containing infant formula enriched with the probiotic strain Lactobacillus fermentum CECT5716. Pharmacol Res;65(2):231-238.

Ishibashi, N., Yamazaki, S. (2001). Probiotics and safety. Am J Clin Nutr; 73:465S-470S.

Jiménez E, Langa S, Martín V, Arroyo R, Martín R, Fernández L, Rodríguez JM. (2010) Complete genome sequence of Lactobacillus fermentum CECT 5716, a probiotic strain isolated from human milk. J Bacteriol. Sep;192(18):4800

Lara-Villoslada, F., Sierra, S., Díaz-Ropero, MP., Rodríguez, JM., Xaus, J., Olivares, M. (2009) Safety assessment of Lactobacillus fermentum CECT5716, a probiotic strain isolated from human milk. J Dairy Res;76(2):216-21.

Maldonado, J., Cañabate, F., Sempere, L., Vela, F., Sánchez, AR., Narbona, E., López-Huertas, E., Geerlings, A., Valero, AD., Olivares, M., Lara-Villoslada, F. (2012) The Human Milk Probiotic Lactobacillus fermentum CECT 5716 Reduces The Incidence of Gastrointestinal And Upper Respiratory Tract Infections In Infants. A Randomised Controlled Trial Comparing A Prebiotic Containing Follow-On Formula Vs The Same Formula Plus Probiotic. J Pediatr Gastroentrol Nutr; 54(1):55-61.

Martin, R., Langa, S., Reviriego, C., Jimenez, E., Marin, ML., Xaus, J., Fernandez, L., Rodriguez, JM (2003). Human milk is a source of lactic acid bacteria to the infant gut. J. Pediatrics. 143: 754-758.

Martin, R., Olivares, M., Marin, ML., Fernandez, L., Xaus, J., Rodriguez, JM. (2005). Probiotic potential of three lactobacilli strains isolated from human breast milk. J. Hum. Lactation. 21: 8-17.

O´Brien, J., Crittenden, R., Ouwehand, AC., Salminen, S (1999). Safety evaluation of probiotics. Trends Food Sci. Technol; 10: 418-424.

Olivares, M., Díaz-Ropero MP., Martín, R., Rodriguez, JM., Xaus, J. (2006) Antimicrobial potential of four Lactobacillus strains isolated from breast milk. J. Appl. Microbiol. 101, 72-79.

Olivares, M., Díaz-Ropero, MP., Sierra, S., Lara-Villoslada, F., Fonollá, J., Navas, M., Rodríguez, JM., Xaus, J. (2007) Oral intake of Lactobacillus fermentum CECT5716 enhances the effects of influenza vaccination. Nutrition;23(3):254-60.

Rodas, AM., Ferrer, S, Pardo, I. (2003) 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. Syst. Appl. Microbiol. 26: 5233-5239.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, WM., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, SE., Mattila-Sandholm, T. (1998). Demonstration of safety of probiotics- A review. Int J Food Microbiol; 44: 93-106.

Saxelin, M., Chuang, NH., Chassy, B., Rautelin, H., Mäkelä, PH., Salminene, S., Gorbach, SL.(1996). Lactobacilli bacteremia in southern Finland, 1989-1992. Clin Infect Dis; 22:564-566.

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