Application for the Authorization of the use of a Rooster Combs Extract in Dairy Products under *Regulation (EC) No 258/97 for the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients.*

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NON-CONFIDENTIAL

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2. EXECUTIVE SUMMARY

BIOIBERICA, S.A. obtains a natural extract from rooster combs, containing glycosaminoglycans, proteins and a high percentage of sodium hyaluronate. Rooster combs have been widely consumed in Europe, being part of several traditional dishes and also being used as a delicacy in many of the high cuisine recipes.

Sodium hyaluronate, the main component of our extract, is a natural substance endogenously found in the intercellular matrix of animal and human connective tissues, as in rooster combs where it is highly concentrated. Sodium hyaluronate is responsible of viscoelastic, lubricating and cushioning properties of joints.

Foods naturally containing sodium hyaluronate are very limited. Only viscera and rooster combs have high amounts of this substance. The maintenance of a varied diet, and also due to cultural habits (not all European countries include rooster combs in their diets), makes difficult to consume these products regularly.

Thus, a good way to make up this lack in sodium hyaluronate could be including a rooster combs extract (RCE) in foods which are daily consumed, like dairy products. Milks or yogurts containing our RCE would supply constant amounts of sodium hyaluronate to our diets, helping our joints to keep in healthy conditions. Also, a dairy product containing the RCE will provide an alternative to the currently marketed food supplements (tablets and capsules which contain high amounts of sodium hyaluronate) and also would be a daily alternative to viscera or rooster combs consumption.

Under *Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients* (hereafter referred to as the Novel Foods Regulation), BIOIBERICA's RCE would be considered as novel when added to a dairy foods. This novel status is due to that a dairy food containing this RCE hasn't been exposed to a significant degree to the EU population prior May 1997.

Therefore, in order to support joint health of the general population, BIOIBERICA, S.A. would like to launch its RCE as a novel food ingredient in dairy products. The proposed recommended daily intake of the RCE would be 80 mg per day.

The specifications of the RCE have been well defined according to the results from the analytical controls performed on the product, demonstrating that the manufacture of the product is homogeneous and provides comparable batches at the end of the process.

BIOIBERICA, S.A. has also performed stability studies on the RCE alone and in a yogurt supplemented with the extract. The results demonstrate that the RCE is stable showing no degradation through the studies lapses (43 months for the long term stability study performed with the extract alone, and 1.5 months for the stability study performed on the yogurt supplemented with the extract).

According to the stability study of the RCE contained in a yogurt, the RCE has shown to be stable also when used in acidic food systems as in dairy products. Consequently BIOIBERICA, S.A. proposes the use of its extract in the following dairy products:

- Milk-based fermented beverages (~3-5 pH)
- yogurts (~3-5 pH)
- Milks (6.8 pH)
- *Fromage frais*

The studies conducted in order to examine the potential toxicity of the RCE have demonstrated that the extract is safe ruling out any toxicity associated to the product.

In vitro genotoxicity test has demonstrated that the RCE is not genotoxic. Furthermore, an acute toxicity study performed with our product demonstrated that the Minimum Lethal Dose of the RCE is greater than 2000 mg/kg when administered orally in rats Sprague-Dawley.

Similarly a 2-weeks-dose-range-finding study and a 4-weeks repetitive administration study showed that the repeated oral administration of the extract to rats at a maximum dose of 600 mg/Kg/day did not produce any noteworthy alteration, since neither mortality nor clinical signs were observed.

A subchronic study showed no noteworthy changes after repeated oral administration of the extract to rats, for 13 weeks at dose levels of 5, 55 and 600 mg/kg.

All of these toxicity results establish the NOAEL (No observed adverse effect level) at 600 mg/Kg/day. For a 60 Kg adult this would be equivalent to up to approximately 5.76 g/capita/day of the RCE, according to the study of Reagan-Shaw *et al,* 2007.

In order to confirm the safety of the product, BIOIBERICA, S.A. has also studied the product in other animal models as horses (Carmona *et al*, 2009) where no adverse events were reported during the study neither significant changes were observed in plasma and synovial fluid analysis.

BIOIBERICA, S.A. has also tested the extract in two human trials (Kalman *et al*, 2008 and Martínez-Puig *et al*, 2009), one of them performed in volunteers who took supplemented yogurts

containing the extract for 3 months. Neither adverse events were reported during the length of both studies (two and three months respectively), nor significant changes were observed in the studied parameters.

The excellent safety and toxicity results coming from the abovementioned studies, allowed BIOIBERICA, S.A. to direct a supplemented food with the RCE to people concerned in maintaining their joints healthy.

Typically target groups include adult population, sport people, elderly and menopause women. Based on the predicted intake of dairy products published by FAOSTAT (Food and Agriculture Organization of the United Nations, FAO), BIOIBERICA, S.A. has calculated the predicted intake of the RCE according to the serving sizes presented in *Table 5.*

Assuming a theoretical situation in which all the dairy products consumed would contain the extract, which would rarely be the case, the intake of the extract would vary within different European countries between 0.26 and 0.66 g per person per day, representing in any case a level of intake superior to a 10% of the established NOAEL.

So, according in all the toxicity and safety studies' results and the stability test reports, it is clear then, that our RCE is a safe and stable product which can be added as a novel food ingredient on dairy products such as milk-based fermented beverages, yogurts, milks or *fromage frais*, at the recommended daily intake of 80 mg/day.

INTRODUCTION

BIOIBERICA S.A. obtains from rooster combs a natural extract, which contains glycosaminoglycans, proteins and a high percentage of sodium hyaluronate.

Sodium hyaluronate is a natural substance endogenously found in the human body and it is responsible of synovial fluid viscoelastic properties, lubricating, cushioning and ensuring a proper function of the joint (smooth, without friction…). It is found in the intercellular matrix of animal connective tissues, such as in rooster combs where it is highly concentrated.

Rooster combs, are nowadays used as part of many dishes in European countries (like France and Spain). It is also used as a typical ingredient in many dishes like homemade chicken soup, stews, several traditional dishes and also used in "haute cuisine" recipes (see Annex V for more examples of the use of rooster combs in nowadays food). It is clear that the rooster combs have an established tradition of safe human consumption in Europe.

BIOIBERICA, S.A. would like to launch its RCE as a novel food ingredient in dairy products (such as fermented-based milk beverages, milks, yogurts and *fromage frais*), contributing with a new alimentary use of rooster combs. This kind of food products containing RCE would help in supplementing our actual diets, lacking of sodium hyaluronate and glycosaminoglycans, due to the decrease in certain animal tissues consumption such as rooster combs.

At the same time, a food product containing this extract would also provide an alternative to the food supplements products currently present in the European market (mostly in Belgium, France, Germany, Ireland, Italy, Portugal, Spain, UK, etc…) which greatly differ on the amounts of sodium hyaluronate.

BIOIBERICA S.A. has prepared this dossier according to *Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients*.

Section 4 of the Commission Recommendation outlines recommendations made by the Scientific Committee for Food (SCF), which facilitates the safety and nutritional evaluation of a given novel food ingredient. Of the six classes identified, BIOIBERICA's RCE would be allocated at Class 2.1 designed as: *A complex (non-GM derived) novel food ingredient, whose source has a history of food use in the Community.*

This dossier for the Application of Authorization of our RCE as a novel food ingredient follows the Commission Recommendation requirements for the application as follows:

I. Specification of the novel food
II. Effect of the production process applied to the novel food
III. History of the organism used as the source of the novel food
IV-VIII. Not applicable as our RCE is not a GM food.
IX. Anticipated intake of use of the novel food
X. Information from previous human exposure to the novel food or its source
XI. Nutritional information on the novel food
XII. Microbiological information on the novel food
XIII. Toxicological information on the novel food

I. SPECIFICATIONS OF THE NOVEL FOOD

This section contains a general description of the principle components of the RCE, the product specifications and the analytical controls.

BIOIBERICA, S.A. performs batch-to-batch analytical controls in order to make sure that the RCE fulfills the specifications. Section *I.3 Specifications compliance*, contains a table showing the results from the analytical controls performed in ten different batches manufactured during 2008/2009.

I.1. GENERAL DESCRIPTION

RCE is the result of the extraction from rooster combs, which have been widely consumed in Europe for years. Nowadays, rooster combs keep on being part of our diets, and are included in products like soups, stews and also as part of sophisticated dishes (See Annex V which contains rooster combs recipes).

RCE consists in a natural extract of rooster combs containing a high percentage of sodium hyaluronate (60-80%), glycosaminoglycans (about 20%) and partially hydrolyzed proteins (about 20%).

SODIUM HYALURONATE, the main component of RCE, is found in the intercellular matrix of animal connective tissues, such as in rooster combs where it is highly concentrated. It was first isolated in 1934 by Karl Meyer from vitreous humour eye. It consists in a linear polysaccharide, whose basic unit is a disaccharide of D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronidic (1-3) bond. The disaccharides units are linearly polymerized by hexosaminidic (1-4) linkages.

CAS Number : 9067-32-7

Chemical name : [→4)-O-(β-D-glucopyranosyluronic acid)-(1→3)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→]

Formula : $[C_{14}H_{20}NNaO_{11}]_n$

Molecular weight : 800,000 Da approximately

Structure :

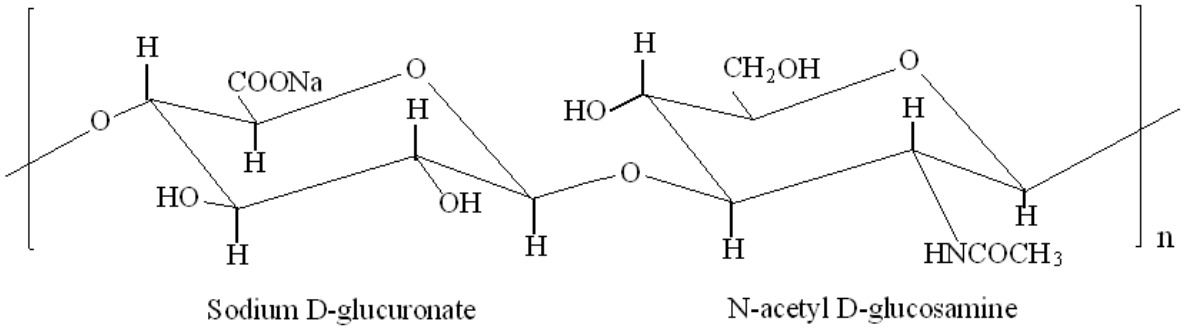

Figure 1. Sodium Hyaluronate molecular structure

GLYCOSAMINOGLYCANS are long unbranched chains of polysaccharides consisting of a repeating disaccharide unit, which form key structural components of the articular cartilage, such as chondroitin sulfate and dermatan sulfate.

HYDROLYZED PROTEINS are polypeptides, peptides and amino acids obtained by the hydrolysis of the proteins already present in RCE, such as hydrolyzed collagen.

Other minority components naturally present in the rooster comb and also in the RCE are minerals like sodium, calcium and phosphorous, which can be found in trace amounts.

I.2. PRODUCT SPECIFICATIONS

Product specifications were set according to the analytical results obtained from the very first 3 manufactured batches. Product specifications are shown in *Table 1. Specifications of the RCE.*

SPECIFICATIONS	LIMITS	METHODS
PHYSICO-CHEMICAL PARAMETERS		
Content in glucuronic, expressed as Sodium Hyaluronate	60 - 80 %	Eur. Ph. Monograph 1472
Appearance	White or almost white hygroscopic powder	Visual
pH	5.0 – 8.5	Eur. Ph. 2.2.3
Chlorides	Not more than 1 %	Mohr Method
Nitrogen	Not more than 8 %	Eur. Ph. 2.5.9
Loss on drying	Not more than 10 %	Eur. Ph. 2.2.32
Heavy metals	Not more than 10 ppm	USP <231>
Mercury	Not more than 0.10 ppm	Eur. Ph. 2.2.58
Arsenic	Not more than 1 ppm	Eur. Ph. 2.2.58
Cadmium	Not more than 1 ppm	Eur. Ph. 2.2.58
Chromium	Not more than 10 ppm	Eur. Ph. 2.2.58
Lead	Not more than 0.5 ppm	Eur. Ph. 2.2.58
Dioxins and furans	Not more than 2.0 pg/g	EPA* Method 1613
PCB's	Not more than 4.0 pg/g	EPA* Method 1613
MICROBIOLOGICAL PARAMETERS		
Total viable aerobic count	Not more than 10^2 cfu/g	Eur. Ph. 2.6.12
Escherichia coli	Absence/ g	Eur. Ph. 2.6.13
Salmonella sp.	Absence/ g	Eur. Ph. 2.6.13
Staphylococcus aureus	Absence/ g	Eur. Ph. 2.6.13
Pseudomonas aeruginosa	Absence/ g	Eur. Ph. 2.6.13

Table 1. Specifications of the RCE

*** EPA:** Environmental Protection Agency

I.3. SPECIFICATIONS COMPLIANCE

Specifications compliance is shown in Tables 2 and 3.

Table 2. RCE analytical results, corresponds to the analysis of 10 different batches of the final product manufactured during 2008 and 2009 including the median and the standard deviation.

As it can be seen on this table, the analytical results for the specification compliance are very stable and homogeneous.

Some parameters like specific heavy metals (mercury, arsenic, cadmium, chromium and lead), dioxins and furans, and PCB's are not analyzed on every single batch. This is due to that the safety and quality of the RCE is well established, so the analysis of these parameters is performed just to assure the absence of these substances twice a year.

Table 3. Statistics results of the 2008 RCE batches, contains the results of the mean, the median, the standard deviation and the standard error of all the analytical results obtained from all batches (n=45) manufactured during 2008.

ANALYTICAL PARAMETERS	SPECIFICATION LIMITS	BATCH ANALYTICAL RESULTS											
		8/0001 Feb 08	8/0010 April 08	8/0021 July 08	8/0038 Nov 08	9/0001 Jan 09	9/0008 Feb 09	9/0009 Mar 09	9/0010 Mar 09	9/0011 April 09	9/0012 April 09	Median	Standard deviation
Sodium Hyaluronate	**60-80 %**	68 %	61 %	65 %	68 %	67.5 %	69 %	65 %	64 %	62 %	67 %	65,5 %	2,58
Appearance	**White or almost white hygroscopic powder**	Almost white	Almost white	Almost white	Almost white	Almost white	Almost white	Almost white	Almost white	Almost white	Almost white	-	-
pH	**5.0 - 8.5**	7.2	7.4	6.7	6.3	6.0	6.6	6.2	6.4	6.5	6.7	6,55	0,432
Chlorides (%)	**Not more than 1%**	0.5	0.7	0.4 %	0.4	0.4	0.5	0.6	0.8	0.5	0.5	0,50	0,134
Nitrogen (%)	**Not more than 8%**	6.0	5.0	7.0 %	7.0	7.0	7.0	7.0	6.0	6.0	6.0	6,50	0,699
Protein by Lowry method (%)	**Not more than 8%**	5.6	4.3	5.2	4.2	4.8	5.2	5.7	6.6	3.6	5.4	5.1	0.86
Loss on drying (%)	**Not more than 10%**	8.3	7.7	5.5	5	5.4	6.0	5.0	6.5	6.0	6.0	6,00 %	1,098
Heavy metals (ppm)	**Not more than 10 ppm**	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	-	-
Mercury (ppm)	**Not more than 0.10 ppm**	< 0.10	-	-	< 0.10	-	-	-	-	-	< 0.10	-	-
Arsenic (ppm)	**Not more than 1 ppm**	< 1	-	-	< 1	-	-	-	-	-	< 1	-	-
Cadmium (ppm)	**Not more than 1 ppm**	< 1	-	-	< 1	-	-	-	-	-	< 1	-	-
Chromium (ppm)	**Not more than 10 ppm**	< 10	-	-	< 10	-	-	-	-	-	< 10	-	-
Lead (ppm)	**Not more than 0.5 ppm**	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	-	-
Dioxins and Furans (pg WHO/TEQ/g)	**Not more than 2.0 pg/g**	0.024	-	-	0.04	-	-	-	-	-	0.07	-	-
PCB's (pg WHO/TEQ/g)	**Not more than 4.0 pg/g**	0.004	-	-	0.006	-	-	-	-	-	0.01	-	-

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ANALYTICAL PARAMETERS	SPECIFICATION LIMITS	BATCH ANALYTICAL RESULTS											
		8/0001 Feb 08	8/0010 April 08	8/0021 July 08	8/0038 Nov 08	9/0001 Jan 09	9/0008 Feb 09	9/0009 Mar 09	9/0010 Mar 09	9/0011 April 09	9/0012 April 09	Median	Standard deviation
Microbiological controls													
Total Aerobic Count (cfu/g)	**Not more than 10^2 cfu/g**	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	≤ 10^2	-	-
E.coli	**Absence/g**	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	-	-
Salmonella	**Absence/g**	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	-	-
Staphylococcus aureus	**Absence/g**	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	-	-
P.aeruginosa	**Absence/g**	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	-	-

Table 2. RCE analytical results

		2008 BATCH ANALYSIS			
PARAMETERS	SPECIFICATION LIMITS	MEAN	MEDIAN	STANDARD DEVIATION	STANDARD ERROR
Sodium Hyaluronate	60-80 %	64,62 %	65,00 %	2,571	0,407
pH	5 - 8.5	6,93 %	6,90 %	0,447	0,071
Chlorides (%)	Not more than 1%	0,50 %	0,50 %	0,148	0,023
Nitrogen (%)	Not more than 8%	6,43 %	6,54 %	0,870	0,138
Loss on drying (%)	Not more than 10%	5,57 %	5,20 %	1,614	0,255

Table 3. Statistics results of the 2008 RCE batches

These statistic results show that, even if the product is an extract and this fact could cause a great variability in the analytical results, there is a good homogeneity among the results due to the low value of the standard deviation and also the low value of the standard error in all analyzed parameters.

I.4. ANALYTICAL PROCEDURES FOR THE ROOSTER COMBS EXTRACT

Next table summarizes the information which can be found in *Annex I. Analytical methods*, which are the analytical controls performed on the RCE. Almost all below mentioned analytical methods are official control methods from the European Pharmacopoeia.

PARAMETERS	REFERENCE	TITLE
CHEMICAL METHODS		
Sodium Hyaluronate	Eur. Ph. Monograph 1472	Sodium hyaluronate
pH	Eur. Ph. 2.2.3	Potentiometric determination of pH
Chlorides	-	Mohr Method
Nitrogen	Eur. Ph. 2.5.9	Determination of nitrogen by sulphuric acid digestion
Loss on drying	Eur. Ph. 2.2.32	Loss on drying
Heavy metals	USP <231>	Heavy Metals
Mercury Arsenic Cadmium Chromium Lead	Eur. Ph. 2.2.58	Inductively coupled plasma-mass spectrometry (ICP-MS)
Dioxins and furans	-	EPA* Method 1613: Tetra-through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS
PCB's	-	EPA* Method 1613: Tetra-through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS
MICROBIOLOGICAL METHODS		
Total viable aerobic count	Eur. Ph. 2.6.12	Microbiological examination of non-sterile products (Total viable aerobic count)
Escherichia coli *Salmonella sp.* *Staphylococcus aureus* *Pseudomonas aeruginosa*	Eur. Ph. 2.6.13	Microbiological examination of non-sterile products (Test for specified micro-organisms)

Table 4. Analytical methods

*** EPA**: Environmental Protection Agency

I.5. ANALYTICAL PROCEDURES FOR THE ROOSTER COMBS EXTRACT IN A YOGURT

For the determination of the presence of RCE in a yogurt, a specific method was developed and validated. This method was used to determinate the presence of sodium hyaluronate in a yogurt by HPLC and can be found in Annex I.

I.6. VALIDATIONS PERFORMED FOR SODIUM HYALURONATE ANALYTICAL METHODS

Annex II contains the validation study of the following analytical method:

- *HPLC determination of sodium hyaluronate in a yogurt*

II. EFFECT OF THE PRODUCTION PROCESS APPLIED TO THE NOVEL FOOD

II.1. DESCRIPTION OF THE MANUFACTURING PROCESS

RCE is obtained by an extraction process from rooster combs, using a mild enzymatic hydrolysis and subsequent filtration, concentration, precipitation and anhydrification. After this process the product will be dried and milled becoming the final white-almost white powder called RCE.

II.1.1. FLOWCHART OF THE ROOSTER COMBS EXTRACT MANUFACTURING PROCESS

II.2. STABILITY OF ROOSTER COMBS EXTRACT

Studies under accelerated storage conditions (40 ± 2ºC / 75 ± 5% Relative Humidity, RH) and long-term storage conditions (25 ± 2ºC / 60 ± 5% RH) have been started with three different production batches of the RCE.

BATCHES TESTED AND CONTAINER CLOSURE SYSTEM:

Three different RCE batches were tested at the two different storage conditions. Each sample was stored in a triple low density polyethylene (LDPE) bag and placed inside a metal drum.

# STUDY	# BATCH	CONDITIONS	DURATION	CONTAINER CHARACTERISTICS
1	5/024	Accelerated Stability	6 months	Triple LDPE bag, closed by cable ties and inside a metal drum
2	6/004	Accelerated Stability	6 months	
3	6/005	Accelerated Stability	6 months	
1	5/024	Long Term Stability	43 months	Triple LDPE bag, closed by cable ties and inside a metal drum
2	6/004	Long Term Stability	40 months	
3	6/005	Long Term Stability	40 months	

TEST AND QUALITY SPECIFICATIONS STUDIED:

The following parameters were selected due to their susceptibility to change with time. These possible changes could affect the quality of the product and, for this reason, they are indicative of the novel food ingredient stability.

TEST	SPECIFICATION
Appearance	White or almost white hygroscopic powder
pH	5.0 – 8.5
Loss on drying	Not more than 10 %
Sodium Hyaluronate	60 – 80 %
Total Viable Aerobic count	Not more than 1000 cfu/g
Moulds and yeasts	Not more than 100 cfu/g
Escherichia coli	Absence/g
Staphylococcus aureus	Absence/g
Salmonella	Absence/g

STUDIES CARRIED OUT:

Accelerated study:

Samples from the three batches to be studied were stored at 40 ± 2ºC / 75 ± 5 % RH in a climatically-controlled chamber. Testing frequency is as follows: 0, 3 and 6 months. As this study is finished, results from the 3-month control are available.

Long-term study:

Samples from the three batches to be studied were stored at 25 ± 2ºC / 60 ± 5 % RH in a climatically-controlled chamber. Testing frequency is as follows: 0, 6, 12, 18, 24, 30, 40 (for batches 6/0004 and 6/0005) and 43 (for batch 5/0024) months. As this study is finished, results from the 40-month and 43-month controls are available.

RESULTS FROM TEST CONTROLS:

Accelerated stability study (40ºC / 75% RH)

Batch 5/024

PARAMETER	INITIAL	3 MONTHS	6 MONTHS
Appearance	Complies	Complies	Complies
pH	6.9	6.4	6.9
Loss on drying (%)	5.6	5.9	5.5
Sodium Hyaluronate (%)	68.0	67.3	65.5
Total Viable Aerobic count	Absence/g	Not determined	Absence/g
Moulds and yeasts	Absence/g	Not determined	Absence/g
Escherichia coli	Absence/g	Not determined	Absence/g
Staphylococcus aureus	Absence/g	Not determined	Absence/g
Salmonella	Absence/g	Not determined	Absence/g

Batch 6/004

PARAMETER	INITIAL	3 MONTHS	6 MONTHS
Appearance	Complies	Complies	Complies
pH	7.1	6.8	6.6
Loss on drying (%)	6.0	6.4	7.3
Sodium Hyaluronate (%)	62.3	61.7	61.0
Total Viable Aerobic count	Absence/g	Not determined	Absence/g
Moulds and yeasts	Absence/g	Not determined	Absence/g
Escherichia coli	Absence/g	Not determined	Absence/g
Staphylococcus aureus	Absence/g	Not determined	Absence/g
Salmonella	Absence/g	Not determined	Absence/g

Batch 6/005

PARAMETER	INITIAL	3 MONTHS	6 MONTHS
Appearance	Complies	Complies	Complies
pH	6.9	6.8	6.8
Loss on drying (%)	5.2	6.0	5.9
Sodium Hyaluronate (%)	61.4	60.0	62.2
Total Viable Aerobic count	Absence/g	Not determined	Absence/g
Moulds and yeasts	Absence/g	Not determined	Absence/g
Escherichia coli	Absence/g	Not determined	Absence/g
Staphylococcus aureus	Absence/g	Not determined	Absence/g
Salmonella	Absence/g	Not determined	Absence/g

Long term stability study (25ºC / 60% RH)

Batch 5/0024

PARAMETER	INITIAL	6	12	18	24	30	43
Appearance	Complies	Complies	Complies	Complies	Complies	Complies	Complies
pH	6.9	6.9	6.9	6.9	6.9	6.3	6.8
Loss on Drying (%)	5.6	6.0	6.4	5.7	6.2	6.2	6.7
Sodium Hyaluronate (%)	68.0	64.3	61.5	62.4	62.1	66.8	63.4
Total Viable Aerobic count	Absence/g	ND	ND	ND	ND	ND	< 10 cfu/g
Moulds/yeasts	Absence/g	ND	ND	ND	ND	ND	< 10 cfu/g
E. coli	Absence/g	ND	ND	ND	ND	ND	Absence/g
S. aureus	Absence/g	ND	ND	ND	ND	ND	Absence/g
Salmonella	Absence/g	ND	ND	ND	ND	ND	Absence/g

ND: Not Determined

Batch 6/004

PARAMETER	INITIAL	6	12	22	30	40
Appearance	Complies	Complies	Complies	Complies	Complies	Complies
pH	7.1	6.8	6.6	6.7	6.7	6.6
Loss on drying (%)	6.0	7.0	7.5	7.4	7.6	6.9
Sodium Hyaluronate (%)	62.3	63.7	63.6	63.1	61.9	62.4
Total Viable Aerobic count	Absence/g	ND	ND	ND	ND	< 10 cfu/g
Moulds and yeasts	Absence/g	ND	ND	ND	ND	< 10 cfu/g
E. coli	Absence/g	ND	ND	ND	ND	Absence/g
S. aureus	Absence/g	ND	ND	ND	ND	Absence/g
Salmonella	Absence/g	ND	ND	ND	ND	Absence/g

ND: Not Determined

Batch 6/005

PARAMETER	INITIAL	6	12	22	30	40
Appearance	Complies	Complies	Complies	Complies	Complies	Complies
pH	6.9	6.8	6.9	6.8	6.7	6.8
Loss on drying (%)	5.2	5.4	5.8	5.6	5.7	5.7
Sodium Hyaluronate (%)	61.4	62.8	62.5	62.3	60.2	62.0
Total Viable Aerobic count	Absence/g	ND	ND	ND	ND	< 10 cfu/g
Moulds and yeasts	Absence/g	ND	ND	ND	ND	< 10 cfu/g
E. coli	Absence/g	ND	ND	ND	ND	Absence/g
S. aureus	Absence/g	ND	ND	ND	ND	Absence/g
Salmonella	Absence/g	ND	ND	ND	ND	Absence/g

ND: Not Determined

INTERPRETATION OF THE STABILITY TEST RESULTS:

Accelerated storage conditions:

The *appearance* of the RCE, aspect and color, did not change after 6 months under accelerated storage conditions for the three tested batches.

The *pH* values maintained between the specifications limits for all three batches and during the whole accelerated stability study.

Loss on drying parameter was softly increased through the time in two of the three batches (6/004, 6/005). However in both cases this parameter remained within the acceptance criteria after 6 months.

No significant changes in *Sodium Hyaluronate* assay have been seen. The Sodium Hyaluronate content has remained inside the specification limits.

Microbiological parameters had excellent results, showing that the product did not develop any microorganism growth during the 6-months accelerated stability study.

Long-term storage conditions:

The *appearance* of the RCE, aspect and color, did not change after 40 and 43 months under long-term storage conditions for the three tested batches.

The *pH* values maintained between the specifications limits for all three batches and during the whole accelerated stability study.

Loss on drying parameter was increased through the time in all three batches. However in all cases this parameter remained within the limits of the specification at the end of the study.

No significant changes in the *Sodium Hyaluronate* content have been seen. The sodium Hyaluronate content have remained inside the specification limits.

All *microbiological parameters* fitted in the specification limits till the end of the study.

CONCLUSIONS:

Exposure to temperatures up to 40ºC ans a relative humidity of 75 % during 6 months, and 25ºC and 60 % of relative humidity during 40-43 months, using as a primary packaging a triple LDPE bag, and a metal drum as a secondary packaging, did not compromise the stability of the RCE.

II.3. STABILITY OF ROOSTER COMBS EXTRACT IN A YOGURT

A study under refrigerated storage conditions was performed with yogurts containing different concentrations of the RCE.

YOGURTS TESTED AND RCE CONCENTRATIONS:

6 different amounts of the RCE were placed in 18 yogurts (3 yogurts for each concentration).

3 more yogurts were used as control thus, no amounts of the extract where added. Concentrations were as follows:

# YOGURTS	RCE CONCENTRATION
3	1.28 mg/g
3	0.96 mg/g
3	0.64 mg/g
3	0.48 mg/g
3	0.30 mg/g
3	0.16 mg/g
3	0 mg/g

STUDIES CARRIED OUT:

In this stability study, two different parameters were controlled:

1. Concentrations of the RCE in yogurts
2. Presence of microorganisms

Testing frequency for both parameters was as it follows: 1 month, and 1.5 months after yogurts preparation.

RESULTS FROM TEST CONTROLS:

Concentration of the RCE in yogurts

Considering a 125 g standard yogurt, concentration marked in red on table below (0.64 mg/g) would represent the recommended daily dosage for our RCE (80 mg). Minor variations on the analytical results occurred when RCE concentrations were analyzed in yogurts after 1 month and 1.5 months of the preparation.

# YOGURT (3 yogurts per concentration)	THEORIC INITIAL CONCENTRATION	1 MONTH	1.5 MONTHS
1 – 3	1.28 mg/g	1.25 mg/g	1.11 mg/g
4 – 6	0.96 mg/g	0.76 mg/g	0.83 mg/g
7 – 9	0.64 mg/g	0.58 mg/g	0.56 mg/g
10 – 12	0.48 mg/g	0.43 mg/g	0.43 mg/g
13 – 15	0.30 mg/g	0.28 mg/g	0.21 mg/g
16 – 18	0.16 mg/g	0.15 mg/g	0.11 mg/g
19 – 21	0 mg/g	< 0.05 mg/g	0.09 mg/g

Presence of microorganisms

1.5 months after yogurts with RCE were prepared, a complete microbiological control was also performed. Results show that microbiological parameters remained according to the limits.

	1.5 Months	
PARAMETER	LIMITS	RESULTS
Moulds	Not more than 10 cfu/g	Complies
Coliforms (n=5 c=2 m=10 M=100)	Complies/g	Complies
Enterobacteriaceae (n=5 c=2 m=10 M=100)	Complies/g	Complies
E. coli (n=5 c=2 m=1 M=100)	Complies/g	Complies
Salmonella (n=5 c=5 m=0)	Complies/25 g	Complies
S. aureus	Not more than 10 cfu/g	Complies
Anaerobic sulphite-reductors	Not more than 1 cfu/g	Complies
Listeria Monocytogenes	Absence/25 g	Complies

CONCLUSIONS:

The analysis of the yogurts containing the RCE after 1 and 1.5 months, perfectly cover the mean shelflife of a standard commercial yogurt, which is normally three weeks. Analysis results show that at 1.5 months, the RCE remained stable even with minor variations, which are considered acceptable, compared to the initial theoretical concentration. Moreover, the presence of the RCE in yogurts did not cause any microbiological presence after 1.5 months.

III. HISTORY OF THE ORGANISM USED AS THE SOURCE OF THE NOVEL FOOD

The novel food ingredient is obtained from an edible non-GM biological source (rooster combs). The source organism is fully characterized and this and/or the food obtained from it are not detrimental to human health.

Rooster combs has had an established human consumption in Europe for ages and continues to be part of the normal diet, including frequently consumed dishes such as home-made recipes (stews) and industrially prepared soup concentrates. First evidences of the use of rooster combs are found in medieval recipes books from 15th century.

Furthermore, rooster combs are also still used as a delicacy in restaurants. See Annex V for more information.

III.1. ROOSTER COMBS EXTRACT SOURCE

Our RCE, is an animal origin product obtained from rooster (*Gallus gallus*) combs declared as fit for human consumption.

Rooster comb is a moderately thin, fleshy formation of smooth soft surface texture, firmly attached from the beak along the top of the skull with a strong base. Rooster comb can measure more than 7 cm and weight more than 8 gr.

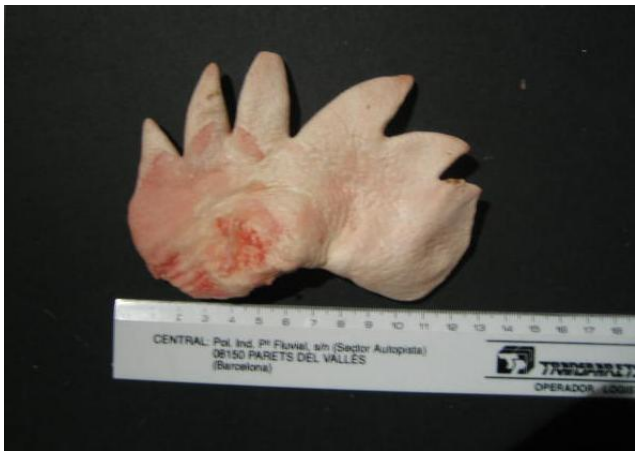

Image 2. Rooster comb

Evidence of the use of rooster combs goes back to medieval recipes in the 15th century, according to the recipes book Platina's On Right Pleasure and Good Health, 1468. (Milham, 1998)

Nowadays, we can find rooster combs as part of our diets incorporated as an ingredient in home-made chicken soup and also in several traditional dishes. In some countries like Spain or France, rooster combs are commonly used as a delicacy (See *Annex V. Rooster combs recipes*), based on an established tradition of consumption in these countries.

Also, culinary recipes can be easily found on the internet.

Packed rooster combs (*Crestas de gallo de corral* in spanish) can also be found as for example in the Spanish Market as shown in the picture below.

Therefore, sodium hyaluronate, glycosaminoglycans and proteins present in rooster combs have been consumed directly by individuals for ages.

Image 2. Packed rooster combs from the Spanish market.
Source: http://www.tiendaargi.es/index.php?main_page=product_info&products_id=16

IX. ANTICIPATED INTAKE OF USE OF THE NOVEL FOOD

RCE could be incorporated in many different food matrices as a novel food ingredient. However, our first example of use proposal is to incorporate it in:

- milk-based fermented beverages
- yogurts
- milks
- *fromage frais*

These products will be taken in one daily serving containing 80 mg of the RCE.

IX.1. PROPOSED USE GROUPS FOR THE USE OF ROOSTER COMBS EXTRACT

The RCE is proposed for the use in yogurts (or any other similar dairy product) for the general population with the exception of pregnant women, children and people allergic to sodium hyaluronate and/or avian proteins.

It is expected that the product would be consumed by adult population, sport people, elderly, and menopause women. The final product containing RCE will be destined to maintain joint health of healthy population with joint discomfort.

Considerations on pregnant women and children

Even it can be deduced from human data we already have that no potentially toxicity risk should be expected when RCE is administered to pregnant women and children, no toxicity studies are yet available for these concrete segment of the population. Due to that, the final product will be labeled for conscious consumers and will contain warnings for pregnant women and children.

Considerations on allergenicity

No allergic episodes have been described in the human and animal studies as a result of the RCE supplementation. As stated in section I.1, RCE contains sodium hyaluronate (60-80%), glycosaminoglycans (about 20%) and partially hydrolyzed proteins (about 20%). Both sodium hyaluronate and glycosaminoglycans have a broad history of use among the UE market (as oral food supplements) without any documented adverse report related to allergenicity. The proteins present in the RCE are partially hydrolyzed, with a mean molecular weight of 1,234 ± 5.1 Da, and for this reason their potential risk of allergenicity is very low.

However, theoretically it could be some cases of hypersensitivity to sodium hyaluronate or avian proteins. Thus, it will also be reasonable to include a warning in the label of the final product containing the RCE for people allergic to sodium hyaluronate and/or avian proteins.

IX.2. INDIVIDUAL PROPOSED FOOD USES AND USE LEVELS

One yogurt containing 80 mg of the RCE per capita and per day is the recommended individual intake established, according to the efficacy studies, in order to appropriately nourishing the joints.

Toxicity studies performed by BIOIBERICA S.A. (see a summary in point *XIII.2. Toxicity studies performed with the RCE*; original copies are enclosed in Annex VI) have shown that the recommended dose of the extract (80 mg/ day) is completely safe.

The historical intake of the original source of the extract, rooster combs, also shows that the recommended dose is safe. The proposed amount of 80 mg daily corresponds to a remarkably inferior quantity of the extract compounds present in a meal portion of rooster combs.

RCE's compounds are present in a comb at an approximate proportion of 1%. So, 25 g of rooster combs (considering a meal portion of 3 combs of approximately 8 g per comb), contain 250 mg of the product, a quantity which is noticeably superior to the recommended daily dose (80 mg).

IX.3. PREDICTED INTAKES

Next table shows the predictable consumption of the RCE in European countries in relation to the human NOAEL. In order to calculate the maximum estimated consumption of the RCE, it has been assumed that all dairy products consumed daily would contain the extract. Predicted total dairy intake for European countries has been obtained from the FAOSTAT (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS) database.

The human equivalent NOAEL (No Observable Adverse Effect Level) has been calculated from the established rat NOAEL (600 mg/Kg/day) and for an average person of 60 Kg, and according to the study of *Reagan-Shaw et al, 2007.*

Thus, as seen on table below, in countries with the highest predicted total dairy intake, like Finland (975.34 g/capita day) or Sweden (1032.88 g/capita/day), the inclusion of the RCE in all dairy products would mean an intake of 0.624 g/capital/day of RCE for Finland and 0.661 g/capital/day for Sweden. In any of the countries the RCE intake would achieve more than a 12% of the NOAEL estimated for humans, again confirming the safety of the RCE even on the worst case scenario.

COUNTRY	PREDICTED TOTAL DAIRY INTAKE EXCLUDING BUTTER*	RCE PREDICTED INTAKE IF INCLUDED IN ALL DAIRIES	% ACHIVED OF THE HUMAN NOAEL (5.76 g/capita/day)**
AUSTRIA	819.18 g/capita/day	0.524 g/capita/day	9.09 %
BELGIUM	701.37 g/capita/day	0.448 g/capita/day	7.77 %
BULGARIA	410.96 g/capita/day	0.263 g/capita/day	4.56 %
CZECH REP.	526.03 g/capita/day	0.336 g/capita/day	5.83 %
DENMARK	654.80 g/capita/day	0.419 g/capita/day	7.27 %
FINLAND	975.34 g/capita/day	0.624 g/capita/day	10.83 %
FRANCE	750.69 g/capita/day	0.480 g/capita/day	8.33 %
GERMANY	698.63 g/capita/day	0.447 g/capita/day	7.76 %
GREECE	715.07 g/capita/day	0.457 g/capita/day	7.93 %
HUNGARY	460.27 g/capita/day	0.294 g/capita/day	5.10 %
ICELAND	684.93 g/capita/day	0.438 g/capita/day	7.60 %
IRELAND	901.37 g/capita/day	0.576 g/capita/day	10 %

This table continues on next page

COUNTRY	PREDICTED TOTAL DAIRY INTAKE EXCLUDING BUTTER*	RCE PREDICTED INTAKE IF INCLUDED IN ALL DAIRIES	% ACHIVED OF THE HUMAN NOAEL (5.76 g/capita/day)**
ITALY	687.67 g/capita/day	0.440 g/capita/day	7.63 %
MALTA	586.30 g/capita/day	0.375 g/capita/day	6.51 %
NETHERLANDS	898.63 g/capita/day	0.575 g/capita/day	9.98 %
NORWAY	726.03 g/capita/day	0.464 g/capita/day	8.05 %
POLAND	473.97 g/capita/day	0.303 g/capita/day	5.26 %
PORTUGAL	586.30 g/capita/day	0.375 g/capita/day	6.51 %
ROMANIA	613.70 g/capita/day	0.392 g/capita/day	6.80 %
SPAIN	473.97 g/capita/day	0.303 g/capita/day	5.26 %
SWEDEN	1032.88 g/capita/day	0.661 g/capita/day	11.4 %
SWITZERLAND	893.15 g/capita/day	0.571 g/capita/day	9.91 %
UNITED KINGDOM	663.01 g/capita/day	0.424 g/capita/day	7.36 %

Table 5. European countries predicted intake of dairy products

* Data from FAOSTAT (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, FAO)

** Human equivalent dose of rat NOAEL (600 mg/Kg/day)

IX.4. PREDICTED INTAKES FOR RISK GROUPS

At the recommended doses, no risk group has been studied.

A dairy product containing the RCE is not addressed to any risk group as pregnant women or children.

X. INFORMATION FROM PREVIOUS HUMAN EXPOSURE TO THE NOVEL FOOD OR ITS SOURCE

Human exposure to the source (Rooster combs):

As already commented, rooster combs, have been widely used in the Community as a food ingredient. Annex V of this dossier contains some of the evidences of its use in Europe.

As RCE's compounds are present in a comb at an approximate 1%, the recommended daily dose (80 mg / day) is inferior to the quantity of the RCE's compounds (250 mg) we can naturally find in a normal portion of combs (25 g, considering 3 combs of 8 g each).

Human exposure to the ingredient (Sodium Hyaluronate):

European market is full of products in form of food supplements, containing this ingredient. These products are sold with no adverse effects reports (See section *X.2 Previous exposure to Sodium Hyaluronate preparations*).

Human exposure to the novel ingredient (RCE):

BIOIBERICA, S.A. has performed trials in humans with the proposed novel ingredient where no adverse effects have been observed (See section *XIII.1.1. Toxicity studies performed with the RCE*).

Therefore, there is plenty of information demonstrating that exposure to the novel food is unlikely to give rise to nutritional, microbiological, toxicological and/or allergenicity problems.

X.1. PREVIOUS ROOSTER COMBS INTAKE

As commented in section *III.1 Rooster combs extract source*, there are lots of evidences which show that rooster combs have been consumed directly by individuals in Europe for years. Therefore, safety of the source of RCE is demonstrated.

It would not be expected that the mild enzymatic digestion, concentration and precipitation to which the rooster combs are subjected would alter the safety of sodium hyaluronate, glycosaminoglycans and hydrolyzed proteins obtained in the extract.

In order to demonstrate the safety of its product, BIOIBERICA, S.A. has performed some toxicological studies, administering intraperitoneal and orally the RCE to rats. Moreover, many other studies demonstrate that no adverse effects, neither allergenicity reactions were observed when studying the efficacy of the RCE administered orally to horses and humans. Section *XIII Toxicological information on the novel food* contains all safety evidences for the RCE.

X.2. PREVIOUS EXPOSURE TO SODIUM HYALURONATE PREPARATIONS

Some examples of the high quantity of food supplements (tablets / capsules) containing Sodium Hyaluronate, present in the European market (mostly Belgium, France, Germany, Ireland, Italy, Portugal, Spain, and UK), are summarized in the following table. Some other product examples are widely explained in Annex VII of this dossier.

BRAND NAME	mg OF SH* PER CAPSULE / TABLET	NUMBER OF CAPSULES / TABLETS RECOMMENDED PER DAY	PRODUCT FORM	SOLD ON
Vital-HA	150 mg	3 capsules (450 mg of SH)	Capsules	On-line
Ultimate HA Formula	150 mg	3 capsules (300 mg of SH)	Capsules	On-line
Synovoderma	210 mg	3 – 6 capsules (from 630 to 1,260 mg of SH)	Capsules	On-line
Puritan's Pride Hyaluronic Acid	100 mg	1 – 4 capsules (from 50 to 200 mg of SH)	Capsules	On-line
Vit.O.Best	36 mg	2 – 4 capsules (from 72 to 144 mg of SH)	Capsules	On-line
Solaray Hyaluronic Acid	60 mg	1 capsule (60 mg of SH)	Capsules	On-line
Hydra-Plenish	100 mg	2 – 4 capsules (from 200 to 400 mg of SH)	Capsules	On-line
Pure HA	100 mg	2 capsules (200 mg of SH)	Capsules	On-line
Skin Eternal Hyaluronic Acid	100 mg	2 tablets (200 mg of SH)	Tablets	On-line
Natrol Hyaluronic Acid	100 mg	4 tablets (400 mg of SH)	Tablets	On-line
Source Natural Hyaluronic Acid	100 mg	2 tablets (200 mg of SH)	Tablets	On-line
Richelet® Anti-Age Peau existe	2.2 mg	1 tablet (2.2 mg of SH)	Tablets	On-line
Hyaluronic Forte®	120 mg	1 tablet (120 mg of SH)	Tablets	On-line

Table 6. Food supplements marketed in the European Union containing sodium hyaluronate

*SH: sodium hyaluronate

As stated in the previous table, Sodium Hyaluronate recommended doses vary from one supplement to another. Almost all of the food supplements containing sodium hyaluronate and present in the market can be easily obtained from the internet. Also, most of supplements do not specify the source of its sodium hyaluronate except “Hydra-Plenish” and “Solaray Hyaluronic Acid” which its source is microbial fermentation.

A dairy product containing Sodium Hyaluronate (as our proposed novel food ingredient, the RCE), would provide an alternative to food supplements at a dose (80 mg/day) lower than the majority of the example products.

XI. NUTRITIONAL INFORMATION ON THE NOVEL FOOD

RCE in dairy products is not intended to replace any food. Nowadays, consumption of rooster combs has been decreased and, as a consequence, also the Sodium Hyaluronate intake. A dairy product containing the RCE would be a good supplementation to our diets. BIOIBERICA, S.A. has performed a trial in humans consuming yogurts supplemented with the RCE (See section *XIII.4. Human Studies*).

Next tables show the nutritional information of a common skimmed yogurt, of the RCE and the nutritional values when the RCE is added to a skimmed yogurt. The result is that the RCE does not alter the profile of the nutrients present in a yogurt, neither the nutritional composition of the yogurt when supplemented with the rooster combs extract.

NUTRITIONAL INFORMATION OF A SKIMMED YOGURT		
PARAMETERS	**Per 100 g of yogurt**	**Per 125 g of yogurt**
Energy	38 kcal 162 KJ	47 kcal 202 KJ
Carbohydrates	4.40 g	5.50 g
Fats	0.10 g	0.10 g
Proteins	4.10 g	5.20 g
Fiber	0 g	0 g
Sodium	56 mg	70 mg

Table 7. Skimmed yogurt nutritional information

NUTRITIONAL INFORMATION OF THE RCE		
PARAMETERS	**PER 100 g**	**PER RECOMMENDED DAILY DOSE (80mg)**
Energy	23.01 kcal / 100 g 95.9 KJ / 100 g	0.02 kcal 0.07 KJ
Carbohydrates	0.49 g	0 g
Fats	0.16 g	0 g
Nitrogen	43.81 g	0.03 g
Fiber	35.7 g	0.02 g
Sodium	2.86 g	0 g

Table 8. RCE nutritional information

When the RCE is added to a skimmed yogurt of 125 g, the nutritional information varies as follows:

PARAMETERS	Per 125 g of yogurt	Yogurt (124.92 g) + Rooster extract (80 mg)	% of Increase
Energy	47 kcal 202 KJ	46.97 kcal 201.94 KJ	- 0.06 % - 0.03 %
Carbohydrates	5.50 g	5.49 g	- 0.18 %
Fats	0.10 g	0.09 g	- 10 %
Proteins	5.20 g	5.22 g	0.38 %
Fiber	0 g	0.028 g	0 %
Sodium	70 mg	72.25 mg	3.11 %

Table 9. Nutritional information of a yogurt containing RCE

As seen in Table 9, the only parameter which is increased when adding the RCE is sodium (3.11 % of increasing regarding the not supplemented yogurt). However, quantities of sodium in the supplemented yogurt are present in such lower quantities of 72.25 mg per 125 g of yogurt.

According to the nutritional information analyzed, the RCE does not alter the general nutritional characteristics of a supplemented yogurt comparing to a not supplemented one.

As seen in the nutritional information, RCE does not supply the yogurt with any relevant nutrient. Moreover, the quantity of RCE added to the yogurt is very low to cause any nutritional impact on an equilibrated diet. It is then not expected that these yogurts supplemented with the RCE will be nutritionally different from those not supplemented.

Next table contains analytical results of 6 industrial yogurts containing 80 mg of the RCE each. We performed the following microbiological controls after 28 days of the manufacturing:

PARAMETERS	YOGURTS					
	#1	#2	#3	#4	#5	#6
Lactobacillus bulgaricus	$44x10^6$ cfu/g	$37x10^6$ cfu/g	$63x10^6$ cfu/g	$46x10^6$ cfu/g	$65x10^6$ cfu/g	$57x10^6$ cfu/g
Streptococcus thermophilus	$36x10^7$ cfu/g	$30x10^7$ cfu/g	$29x10^7$ cfu/g	$39x10^7$ cfu/g	$31x10^7$ cfu/g	$29x10^7$ cfu/g

Table 10. Microbiological information of a yogurt containing RCE

As seen in Table 10, microorganisms naturally present in yogurts like *Lactobacillus bulgaris* and *Streptococcus thermophilus* maintain their presence even the addition of the RCE.

So, it is demonstrated that the RCE does not alter the nutritional characteristics of a supplemented dairy product like a yogurt.

XII. MICROBIOLOGICAL INFORMATION ON THE NOVEL FOOD

There is no presence of any microorganisms or their metabolites due to the novelty of the novel food ingredient.

The manufacturing extraction process of the RCE is based in the Good Manufacturing Practices and moreover, BIOIBERICA, S.A. controls the presence of any microorganism (virus and bacteria) which could alter the quality and safety of the product.

Table 1 of section *I.2 Product Specifications,* summarizes all parameters controlled and the methods used. Appropriate limits for total viable aerobic count and reference pathogen micro-organisms are established and complied with.

Microbiological controls are conducted on a batch-to-batch basis in order to guarantee that the manufacturing process is capable to eliminate any potential bacterial contamination present in the raw material and to prevent bacterial growth during production.

RCE is obtained from animals declared fit for human consumption (See Health Certificate attached in Annex III). Careful selection of this animal raw material provides a measure of confidence that the final product is safe as regards potential viral contamination. Nevertheless, the manufacturing process is performed under specific conditions that are efficient in the inactivation/removal of several conventional viruses.

Annex VIII contains the final report of a study which evaluates the clearance of MLV, influenza, REO3 and PPV by the purification process for the RCE for the ability to eliminate adventitious and endogenous virus contaminants. This study was performed by BioReliance Invitrogen Bioservices, in September 2006, and designed to comply with the CPMP Note for Guidance on Virus (models) Validation Studies. The study concludes that different steps of the purification process of our RCE, concretely the enzymatic digestion, solvent precipitation and H_2O_2 treatment, were effective in the inactivation/removal of viruses considered as models.

As for a yogurt supplemented with the RCE, section *II.2 Stability of the RCE in a yogurt,* contains the microbiological controls 1.5 months after the yogurt preparation, demonstrating that the addition of the RCE to the yogurt does not promote the presence of any pathogenic microorganism.

XIII. TOXICOLOGICAL INFORMATION ON THE NOVEL FOOD

BIOIBERICA, S.A. has studied the safety and efficacy of the proposed novel food ingredient in various different trials (See section *XIII.2. Toxicity studies performed with the RCE, section XIII.3. Other animal studies with the RCE and section XIII.4. Human studies.*

There is no information suggesting that the novel food might pose allergenic risk to humans. None of the trials performed in humans or in animals have reported any adverse effect or allergenic reactions.

XIII.1. TOXICOLOGICAL ASSESSMENT

RCE is a demonstrated safe product. Many analytical and toxicity studies show its harmlessness.

Next three tables summarize the analytical results of the presence of heavy metals, dioxins, furans and PCB's in the RCE:

HEAVY METALS ANALYSIS

RCE BATCH	MERCURY	ARSENIC	CADMIUM	CHROMIUM
8/0001	< 0.10 ppm	< 1 ppm	< 1 ppm	< 10 ppm
8/0038	< 0.10 ppm	< 1 ppm	< 1 ppm	< 10 ppm
9/0012	< 0.10 ppm	< 1 ppm	< 1 ppm	< 10 ppm

Table 11. Analytical results for heavy metals

DIOXINS AND FURANS ANALYSIS

RCE BATCH	pg WHO / TEQ / g
8/0001	0.024
8/0038	0.04
9/0012	0.07

Table 12. Analytical results for dioxins and furans

PCBs ANALYSIS

RCE BATCH	pg WHO / TEQ / g
8/0001	0.004
8/0038	0.006
9/0012	0.01

Table 13. Analytical results for PCB's

XIII.2. TOXICITY STUDIES PERFORMED WITH THE ROOSTER COMBS EXTRACT

Next table contains a summary of the toxicity studies performed with the RCE in rats. Moreover, we have included, as a support, some efficacy studies of the product in horses and in humans, showing the safety and the harnessless of the product. The last study included in the table below was performed in humans, studying the safety and efficacy of our RCE as an ingredient in yogurts, so in the matrix where we would like to commercialize our product. According to all the results from the toxicity studies conducted with the RCE, and the history of safe use of its raw material (rooster combs), we can state that our RCE is a safe novel ingredient.

TITLE OF THE STUDY	TYPE	SUBJECT STUDIED	ADMIN. ROUTE	DOSIFICATION	SAFETY CONCLUSIONS
Genotoxicity test	In vitro	*Salmonella* *E.Coli*	-	Five concentrations	• No toxicity in any of the strains • No mutagenic responses
Acute oral toxicity in rat	In vivo	18 rats	Orally (gastric gavage)	1,000 mg/Kg 2,000 mg/Kg	• No mortality at 2000 mg/Kg • No clinical signs during or after treatment
2-week dose-range-finding study	In vivo	40 rats	Orally (gastric gavage)	200 mg/Kg/day 400 mg/Kg/day 600 mg/Kg/day	• No mortality neither alterations in feed consumption, body weight or necropsies • No clinical signs observed.
Oral toxicity by 4-weeks repetitive administration	In vivo	100 rats	Orally (gastric gavage)	5 mg/Kg/day 55 mg/Kg/day 600 mg/Kg/day	• No mortality neither alterations in feed consumption, body weight or necropsies • No clinical or histological signs observed.
13-week oral (gavage) toxicity in rats with a 4-week recovery period	In vivo	100 rats	Orally (gastric gavage)	5 mg/Kg/day 55 mg/Kg/day 600 mg/Kg/day	• No mortality neither alterations in feed consumption, body weight or necropsies • No clinical or histological signs observed.

This table continues on the next page

TITLE OF THE STUDY	TYPE	SUBJECT STUDIED	ADMIN. ROUTE	DOSIFICATION	SAFETY CONCLUSIONS
Acute intraperitoneal toxicity in rat	In vivo	26 rats	Intraperitoneally	250 mg/Kg/day 500 mg/Kg/day 900 mg/Kg/day 1000 mg/Kg/day	• No mortality observed. • Observed clinical signs post administration as abnormal locomotion, piloerection. • Minimum Lethal Dose of the RCE established in more than 1000 mg/Kg
Study of the intestinal absorption of the RCE	In vitro	6 rats	-	Solution of 200 µg/ml of the RCE	• The RCE is absorbed from the media through the intestinal mucous. • The most important absorption occurs in the duodenum.
Study of the effects of the RCE on Hyaluronic Acid concentration in a horse model. (60 days administration)	In vivo	12 horses	Orally	250 mg/day	• Treated horses presented higher levels of hyaluronate in the synovial fluid. Any adverse reaction was observed or any clinical sign. No lameness neither joint effusion occurred.
Clinical Trial on efficacy and safety of the RCE. (8 weeks administration)	In vivo	20 adults	Orally	80 mg/day	• No serious adverse events were reported. • The RCE appeared to be well tolerated and safe. • No alteration son body weight, vital signs, and safety laboratory results.
Clinical trial evaluating the efficacy and safety of a yogurt supplemented with the RCE	In vivo	40 adults	Orally	80 mg/day	• No significant changes in body weight or clinical parameters as pulse rate or blood pressure were observed.

Table 14. Summary of all Toxicity and Efficacy studies performed with the RCE

XIII.2.1. GENOTOXICITY TEST

This bacterial reverse mutation study was carried out by RCC CIDA S.A. in Barcelona, Spain. The study was performed following Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 2004/10/EC.

The study begun in January 2008 and the final report (No.S11430) was signed in July 2008. See Annex V for the complete study.

STRAINS USED

Ames strains TA-1535, TA-1537, TA-98 and TA-100 of *Salmonella typhimurium* and *Escherichia coli* WP2 uvra pkM101 were treated with BIOIBERICA's RCE, at five different concentrations. The aim of this study was to assess the possible mutagenic potential of the RCE in presence and absence of a metabolic activation system.

PROCEDURES

Bacterial preparation:

Five bacterial strains were defrosted and grown on Master plates in order to obtain pure cultures. These plates were stored between 2 and 8 ºC. Sixteen hours before each test, an inoculums was prepared for each of the five bacterial strains in 20 ml of nutrient broth and was incubated in a bath a 37 ± 1 ºC with agitation.

Test item preparation:

Dimethylformamide was used as a solvent for the test item (RCE). 100 mg of the extract was wighed and dimethylformamide was gradually added.

Metabolic activation system preparation:

Homogenized rat liver (S-9), induced with Aroclor was used to check whether the test item was active when metabolized. S-9 was used at a concentration of 10% in standard cofactors.

Experimental procedure:

0.1 ml of test item or the positive control were preincubated with 0.1 ml of the strain culture and 0.5 ml of sterile phosphate buffer or of metabolic activation system for 20 minutes at 37 ± 1ºC. Plates were incubated for 72 hours at 37 ± 1ºC. After the incubation period, the number of

relevant colonies that had grown on each plate was counted. The whole experiment was repeated another day, using fresh solutions and fresh bacterial cultures.

Positive control:

Parallel to the study of the test item, known mutagenic products were tested in order to check the sensitivity of mutagenic agents of the strains used.

Negative control:

The solvent used to dilute the test item, dimethylformamide, was tested as the negative control.

STATISTICAL ANALYSIS

The comparisons between the results for the reference item and the control were made using Student's *t* test with levels of significance of $p < 0.01$ and $p < 0.05$. The statistical comparison of the different test-item concentrations and the control was carried out, for all the bacterial strains, both with and without metabolic activation, using a one-way analysis of variance with a level of significance of $p < 0.05$.

RESULTS

- All bacterial strains responded positively when treated with the positive controls. Accordingly, normal values were obtained in the revertant colony counts on all the plates treated only with the solvents (negative controls).
- About the test item, the RCE did not cause toxicity in any of the strains. No mutagenic response was observed in any of the tested strains, with or without the S-9.

CONCLUSIONS

- Based on the results, it may be concluded that the test item RCE, under the experimental conditions used produced no mutagenic activity in any of the five bacterial strains used.

XIII.2.2. ACUTE ORAL TOXICITY IN RAT

The Acute Oral Toxicity test on the RCE was carried out by the *Centro de Investigación y Desarrollo Aplicado, S.A.L.* (CIDASAL) in Barcelona, Spain. The study was performed following Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 1999/11/EC.

The study was begun in March 2004 and the final report (No. CD04/9053T) was signed in May 2004. See Annex V for the complete study.

ANIMALS USED

18 rats Sprague Dawley “SD” (9 male and 9 female) were used in the study. The animals in the Preliminary Study weighed between 110-141 g and those used in the Principal Study weighed 137–150 g and 119–131 g for the male and female rats, respectively.

The rats were housed in Makrolon cages (59.0 x 38.5 x 20.0 cm) in groups of maximum 5 rats of the same gender during the period of acclimatation. The cages were identified by means of a card indicating the number of the study and the housed animals, their sex, code of the tested product, dose, administration route, date of administration, date of entrance of the animals and person in charge of the study. The cages were distributed on the shelves in order to equilibrate any external factor (environmental conditions) that could have an influence on the animals.

The study was performed at a temperature of 20-25ºC, and a relative humidity of 30-65%.

The illumination was artificial and controlled to provide 12 hours of light (7:00 to 19:00 h) and 12 hours of darkness.

DIET

The animals had free access to a standard diet for rats SAFE A04C.

WATER

The animals had water *ad libitum* provided with bottles. The water is analyzed periodically to detect the presence of potential contaminants.

ROUTE OF ADMINISTRATION, FREQUENCY AND VOLUME

The test product, the RCE codified as IB0004, was administered orally by means of a gastric sonde. The administration was carried out in one time, with a volume of 10 ml/kg for the dose of 1000 mg/kg. The dose of 2000 mg/kg was administered fractionated, allowing a period of 4 hours between the first and second administration, with a volume of 20 ml/kg.

The volume of administration to each animal was determined according its weight at the moment of administration.

The rats administered with the dose of 1000 mg/kg were fasted for about 18 hours before the administration, and the rats administered with the dose of 2000 mg/kg for 12-16 hours.

All the animals were offered food 3-4 hours after the treatment.

DOSE LEVEL AND GROUP SIZE

Preliminary Study

The RCE was administered as follows:

TREATMENT	DOSE (mg/Kg)	No ASSIGNED TO THE ANIMALS	
		Male	Female
RCE (IB0004)	1000	1, 2	3, 4
	2000	5, 6	7, 8

Principal Study

The RCE was administered as follows:

TREATMENT	DOSE (mg/Kg)	No ASSIGNED TO THE ANIMALS	
		Male	Female
RCE (IB0004)	2000	9 to 13	14 to 18

OBSERVATIONS

Preliminary Study

After the administration, the rats were observed at least twice a day for 14 days. After this period, the animals were sacrificed.

Principal Study

During the day of the administration, the rats were observed frequently, in order to detect clinical signs. Additionally, the animals were observed at least twice a day for 14 days.

The observations included, at least, changes in the skin, hair, eyes and mucous membranes, in the respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

After this period of observation, the animals were sacrificed.

Body weight

All the rats were weighed before the administration and at half of the period of observation. Before their sacrifice, the animals were again weighed.

Sacrifice and *post-mortem* procedures

After the period of observation, all the animals were sacrificed by intraperitoneal injection of sodium pentobarbital, performing the necropsy of all the animals from the Principal Study.

The necropsy included the assessment of the intact animal and all of the superficial tissues, followed by the observation of the cranial, thoracic and abdominal cavities.

RESULTS

Preliminary Study

- None of the animals administered with the RCE at a dose of 1000 and 2000 mg/kg died.
- No clinical signs were observed in the same animals neither during the day of the administration nor during the days following the treatment.
- The evolution of the corporal weight of all the animals was normal.

Principal Study

- None of the animals administered with the RCE at a dose of 2000 mg/kg died.
- No clinical signs were observed in any animal treated with a dose of 2000 mg/kg, neither during the day of the administration nor during the days following the treatment.
- The evolution of the corporal weight of all the animals was normal.
- One male and one female showed a dark area in the stomach wall of about 1 and 5 mm of diameter, respectively. The rest of the necropsys didn't show macroscopic alterations.

CONCLUSION

- No mortality was observed at the highest dose administered, 2000 mg/kg. This dose is determined by the maximum concentration of the product soluble in distilled water that allows a formulation suitable for its oral administration in rats at the maximum volume of administration of 20 ml/kg.
- Consequently, considering the dose of 2000 mg/kg high enough, the Maximum Non-Lethal Dose and the Minimum Lethal Dose, were not determined.
- According to the results obtained, the Minimum Lethal Dose of the RCE is greater than 2000 mg/kg when administered orally in rats Sprague Dawley.

XIII.2.3. 2-WEEK DOSE-RANGE-FINDING STUDY

This test on the RCE was carried out by the Centro de Investigación y Desarrollo aplicado, S.A.L. (CIDASAL) in Barcelona, Spain. The study was performed following the Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 2004/10/EC.

The study was begun in November 2004 and the final report (No. CD04/9438T) was signed in February 2005. See Annex V for the complete study.

ANIMALS USED

40 rats Wistar Hannover HsdBrlHan:WIST (20 males and 20 females) were used in the study. Medium weight of the animals was 134g for males, and 123 g for females.

The animals were housed in Makrolon cages (59.0 x 38.5 x 20.0 cm) in groups of maximum 5 rats of the same gender. Each cage was identified by a card, colour-coded according to the dose level. This card indicated the number of the cage, number and sex of the animals housed, number of the study, test item code, administration route, dose, name of the Study Director, and date of arrival of the animal and of the start of treatment.

The study was performed at a temperature of 22 ± 3 ºC and a relative humidity of 40-75%.

The lighting in the animal housing was controlled to give 12 hours of light (7:00 to 19:00) and 12 hours of darkness (19:00 to 7:00) every 24 hours.

The cages were arranged on the shelving so that external factors, such as environmental conditions, were balanced as much as possible.

DIET

The animals had free access to a standard rodent diet SAFE A04C.

WATER

The animals had water *ad libitum* provided with bottles. The water was analyzed periodically to detect the presence of potential contaminants.

ROUTE OF ADMINISTRATION, FREQUENCY AND VOLUME

The test item, the RCE (IB0004), was administered orally by gastric gavage.

The animals were administered once a day, seven days a week, during 2 weeks with a volume of 20 ml/Kg.

The volume of administration to each animal was determined according its weight at the moment of administration.

The rats from the Control group were treated only with the vehicle (distilled water), at the same administration volume as the rest of the treatment groups.

DOSE LEVELS AND GROUP SIZE

The study was conducted with four groups, including the control group, and rats were distributed in the following way:

GROUP		1	2	3	4
Tested product		Control	IB0004	IB0004	IB0004
Dose (mg/kg/day)		-	200	400	600
Colored code		White	Blue	Green	Red
Number	Males	1 to 5	6 to 10	11 to 15	16 to 20
	Females	21 to 25	26 to 30	31 to 35	36 to 40

- Doses:

- Due to the characteristics of the test item, the dose levels were selected based on the maximum dose administrable by oral route (600 mg/kg day).

OBSERVATIONS

Mortality

Two rats found dead during the study underwent the necropsy procedure (see Sacrifice and macroscopic examination).

Clinical signs

All rats were observed in great detail at least twice a day, in order to record any symptom of illness or reaction to the treatment.

The observations, also made at the weekends, included at least, changes in the skin, fur, eyes and mucous membranes, in the respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral.

Body weight

The body weight of each rat was recorded before the start of treatment, daily during the treatment period, and before sacrifice.

Food intake

The food intake per cage was recorded before the start of treatment, and then once a week after that, and the mean weekly intake per rat was calculated.

SACRIFICE AND MACROSCOPIC EXAMINATION

At the end of the second week of treatment, all rats were sacrificed by means of exsanguination after deep anesthesia with pentobarbital sodium by intraperitoneal route.

A full autopsy was carried out on all animals. This included an examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities, and their contents both *in situ* and after evisceration.

Following the macroscopic examination, some organs were weighed and some other organ samples were taken for a histological examination.

STATISTICAL ANALYSIS

The body weights and organs weights were evaluated by means of a one-factor analysis of variance. When statistically significant differences were found, the differences among groups were evaluated using Dunnett's method ($p<0.05$ and $p<0.01$).

RESULTS

- No mortality or clinical signs attributable to the treatment among the animals treated with the RCE.
- Body weight of the animals treated with the RCE and the animals of the Control group had no alterations along the period of treatment.
- Food intake among the animals treated with the RCE wasn't affected by the treatment, and it was similar to the Control group. The exception was the group administered at 200 mg/kg/day that had a higher food intake than the Control group, but this happened even at the beginning of the treatment.
- Necropsies of the sacrificed animals, showed only in some animals treated at doses of 400 mg/kg/day and 600 mg/kg/day, some reddish coloring on the thymus and on the mandibular lymph nodes. There were no gross lesions that could be attributed to the test item. All findings recorded were considered to be within the range of normal background lesion, which may be seen in rats of this strain.
- No other macroscopic alterations were observed in necropsies, neither differences in the organ weight among the animals treated with the RCE.

CONCLUSION

- The repeated administration of the test item, the RCE, to rats during a period of two consecutive weeks, at the doses of 200, 400 and 600 mg/Kg/day did not produce any noteworthy alteration, since neither mortality or clinical signs were observed and there were no differences in body weights in comparison with Control group.
- The only thing observed was a reddish colouring on the mandibular lymph nodes and reddish areas on the thymus, in some animals from the high and intermediate doses.
- Based on the results obtained, the dose of 600 mg/kg/day is proposed as the high dose in the 4-week study, since it was the maximum dose that could be administered by oral route.

XIII.2.4. ORAL TOXICITY BY 4-WEEKS REPETITIVE ADMINISTRATION

The Oral Toxicity test on the RCE was carried out by the Centro de Investigación y Desarrollo aplicado, S.A.L. (CIDASAL) in Barcelona, Spain. The study was performed following the Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 2004/10/EC.

The study was begun in March 2005 and the final report (No. CD04/9491T) was signed January 2006. See Annex V for the complete study.

ANIMALS USED

100 rats Wistar Hannover HsdBrlHan:WIST (50 males and 50 females) were used in the study. Medium weight of the animals was 103 g for males, and 99 g for females.

The animals were housed in Makrolon cages (59.0 x 38.5 x 20.0 cm) in groups of maximum 5 rats of the same gender. Each cage was identified by a card, colour-coded according to the dose level. This card indicated the number of the cage, number and sex of the animals housed, number of the study, test item code, administration route, dose, name of the Study Director, and date of arrival of the animal and of the start of treatment.

The cages were arranged on the shelving so that external factors, such as environmental conditions, were balanced as far as possible.

The study was performed at a temperature of 22 ± 2 ºC and a relative humidity of 30-60%.

The lighting in the animal housing was controlled to give 12 hours of light (7:00 to 19:00) and 12 hours of darkness (19:00 to 7:00) every 24 hours.

DIET

The animals had free access to a standard rodent diet SAFE A04C.

WATER

The animals had water *ad libitum* provided with bottles. The water was analyzed periodically to detect the presence of potential contaminants.

ROUTE OF ADMINISTRATION, FREQUENCY AND VOLUME

The tested product, the RCE, was administered orally by gastric gavage.

The administration was carried out once a day, seven days a week, during 4 weeks with a volume of 20 ml/Kg.

The volume of administration to each animal was determined daily according its weight at the moment of administration.

The rats from the Control group were treated only with the vehicle (distilled water), at the same administration volume as the rest of the treatment groups.

DOSE LEVEL AND GROUP SIZE

The study was performed with four groups, including the control group, and rats were distributed in the following way:

GROUP		1	2	3	4
Tested product		Control	RCE	RCE	RCE
Dose (mg/kg/day)		-	5	55	600
Colored code		White	Blue	Green	Red
Sacrifice at the end of treatment	Males	1 to 10	16 to 25	26 to 35	36 to 45
	Females	51 to 60	66 to 75	76 to 85	86 to 95
Sacrifice after recovery period	Males	11 to 15	-	-	46 to 50
	Females	61 to 65			96 to 100

- Doses:
  - High dose of 600 mg/Kg/day was selected to be the maximum oral dose administrated due to the solubility characteristics of the product.
  - Low dose of 5 mg/Kg/day was selected as a lower multiple of the foreseen dose for humans (0.66 mg/Kg/day approximately).

- Intermediate dose of 55 mg/Kg/day was selected as a geometric medium dose between the maximum and the minimum doses.

RECOVERY PERIOD

Once concluded the period of treatment, 5 males and 5 females from control group and from the maximum dose treatment group, were subjected to a 2 weeks recovery period where the animals didn't have any administration.

The objective of this recovery period was to study the reversibility or the evolution of possible alterations registered.

OBSERVATIONS

Mortality:

All rats were observed daily just to detect clear signs of morbidity or mortality.

Clinical signs:

All rats were observed in great detail at least once a day, in order to record any symptom of illness or reaction to the treatment.

The observations included at least, changes in the skin, fur, eyes and mucous membranes, in the respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral.

Body weight:

The body weight of each rat was recorded before the start of treatment, daily during the treatment period, and before sacrifice.

Those rats subjected to the 2 weeks recovery period were weighed twice a week.

Food intake

The food intake per cage was recorded one week before the start of treatment, and then once a week after that, and the mean weekly intake per rat was calculated.

Ophthalmoscopy:

The eyes of all the animals were examined before the beginning of the treatment.

During the last week of treatment, the eyes of the animals belonging to the control group and to the maximum dose group were examined.

Later, during the last week of the recovery period, the eyes of the animals belonging to these groups were examined again.

The examination was carried out by means of eye drops of cyclopentolate chlorohydrate at 1% in order to dilate the pupils.

Laboratory studies:

During the fourth week of treatment, blood samples were extracted from 10 males and 10 females of each group. These animals didn't have any access to food during 16 hours previous to the extraction.

Blood samples of each animal were extracted between 7:30 and 10:00 am, in order to reduce the biological variation caused by circadian rhythms.

Also, urine samples were collected during 16 hours, of the same 10 males and 10 females of each group.

Later, during the last week of the recovery period blood and urine samples of all animals subjected to this recovery period were analyzed.

SACRIFICE AND MACROSCOPIC EXAMINATION

By the end of the fourth week of treatment, all rats were sacrificed by means of exsanguination after deep anesthesia with pentobarbital sodium by intraperitoneal route, except from those rats subjected to the recovery period that were sacrificed at the end of this.

A full autopsy was carried out on all animals. This included and examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities, and their contents both *in situ* and after evisceration.

Following the macroscopic examination, some organs were weighed and some other organ samples were taken for a histological examination.

HISTOLOGICAL EXAMINATION

The aim of the microscopic observations was to examine organs and tissues from those animals that some adverse event was observed:

- Tissues from dead animals, in order to find out the cause of the death.
- Tissues from all the animals belonging to the Control group and to the maximum dose group that were sacrificed at the end of the treatment.
- All the organs and tissues of the animals sacrificed at the end of the recovery period from those animals that some effect was observed in the treatment group.

STATISTICAL ANALYSIS

The body weights and organs weights were evaluated by means of a one-factor analysis of variance ANOVA ($p<0.05$ and $p<0.01$). When statistically significant differences were found, the differences among groups were evaluated using Dunnett's method ($p<0.05$ and $p<0.01$).

The rest of urinary parameters were statistically evaluated with an homogeneity test (Chi2 $<p<0.05$).

RESULTS

- No mortality and clinical signs attributable to the treatment among the animals treated with the RCE.
- Body weight of the animals treated with the RCE and the animals of the Control group had no alterations along the period of treatment.
- Food intake among the animals treated with the RCE had no differences from the Control group during the treatment period neither during the recovery period.
- There were no ocular alterations related to the treatment.
- There weren't any alterations attributed to the treatment in the hematological and urinary analysis during the fourth week of treatment, neither during the recovery period.
- No macroscopic alterations attributed to the treatment were observed in necropsies neither at the end of the treatment, nor at the end of the recovery period between the animals treated with the RCE.
- No significant alterations were observed on the organs weight, only a decrease of thymus weight among the females treated at a dose of 600 mg/kg/day.

- There were neither injuries in the histological examination related to the administration of the RCE among the animals sacrificed after the period of treatment, nor after the recovery period.

CONCLUSION

- The repeated administration of the test item, the RCE, in rats during four consecutive weeks, at doses of 5, 55 and 600 mg/Kg/day did not produce any noteworthy alteration, since neither mortality or clinical signs were observed in any of the treatment groups.
- Histological examination of the animals treated with the RCE showed no alterations attributed to the treatment.
- According to the results obtained, the NOAEL (No Observable Adverse Effect Level) may be established at 600 mg/Kg/day for the oral administration to rats during a period of 4 weeks.

XIII.2.5. 13-WEEK ORAL (GAVAGE) TOXICITY STUDY IN RATS WITH A 4-WEEK RECOVERY PERIOD

The Sub-chronic Oral Toxicity test was carried out by Harlan Laboratories S.A. in Barcelona, Spain. The study was performed following the Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 2004/10/EC.

The study was begun in June 2008 and the final report (No.S11970) was signed in June 2009. See Annex V for the complete study.

ANIMALS USED

100 rats Wistar Hannover HsdBrlHan:WIST (50 males and 50 females) were used in the study. Body weight range of the animals was 205.2 – 250.4 g for males, and 148.9-182.0 g for females and age of both genders was between 7-9 weeks.

The animals were housed in Makrolon type-5 cages with sawdust bedding Lignocel 3-4, in groups of maximum 5 rats of the same gender. Each cage was identified by a card, and a tail or ear tattoo mark. The study was performed at optimum hygienic conditions behind a barrier system, air-conditioned with a minimum of 10-15 air changes per hour, and continuously monitored environment with target for temperature approximately 22 ± 3 ºC and 12 hours fluorescent light/12 hours of dark.

The group identification and animal numbers assigned to treatment are stated in the following table:

GROUP	1	2	3	4
Tested product	Control	RCE	RCE	RCE
Dose (mg/kg/day)	0 mg/kg/day*	5 mg/kg/day	55 mg/kg/day	600 mg/kg/day
Males A	1-10	16-25	26-35	36-45
Males B**	11-15	-	-	46-50
Females A	51-60	66-75	76-85	86-95
Females B**	61-65	-	-	96-100

A: Main Study (termination after 13 weeks of treatment).
B: Recovery (termination after 13 week of treatment and 4 weeks of recovery).
*: Control animals were treated with vehicle only.
**: According to *OECD Guideline for the Testing of Chemicals Guideline 452 Chronic Toxicity Studies, 12 May 1981*, two satellite groups are included to monitor the reversibility of any toxicological changes induced by the chemical under investigation (control and higher dose).

DIET

Standard dry pelleted diet for rodents Harlan Teklad 2014C rat/mouse maintenance diet *at libitum*. The composition of each batch is analyzed as well as checked for contaminants.

WATER

The animals had access *ad libitum* to community tap water provided by Compañía de Aguas Sabadell S.A.. The water is analyzed periodically to detect the presence of potential contaminants.

ROUTE OF ADMINISTRATION, FREQUENCY AND VOLUME

The tested product, BIOIBERICA's RCE, was administered orally by gastric savage. The administration was carried out once a day, seven days a week, during 13 weeks with a volume of 20 ml/Kg. The rats from the Control group were treated only with the vehicle (distilled water), at the same administration volume as the rest of the treatment groups.

DOSE LEVEL AND GROUP SIZE

Dose level was based on the results obtained from a 4-week toxicity (gavage) study with the test item in rats (*4-week toxicity study in rats with repeated oral administration and a 2-week recovery period*). The study was performed with four groups, including the control group, and rats were distributed in the following way:

GROUP	1	2	3	4
Tested product	Control	RCE	RCE	RCE
Dose (mg/kg/day)	-	5 mg/kg	55 mg/kg	600 mg/kg

- <u>Doses:</u>
  - The high dose (600 mg/kg) was selected because it is the maximum administrable dose by oral route.
  - The low dose (5 mg/kg) was selected because it is a low multiple of the intended human dose (approximately 0.66 mg/kg).

- The intermediate dose was selected because it is the geometric mean between the high and low doses.

RECOVERY PERIOD

Once concluded the period of treatment, 5 males and 5 females from group 1 and 4 underwent a 4-week treatment-free period. This recovery period corresponded to weeks 14 and 17, inclusive, of the Study, considering the first week of treatment as the first week of the Study.

The objective of this recovery period was to study the reversibility or the alterations observed during the treatment period.

OBSERVATIONS

Viability / Mortality

All rats were observed daily just to detect clear signs of morbidity or mortality. Moribund animals and those presenting severe clinical signs were sacrificed for ethical reasons and to avoid autolysis of the tissues.

Clinical signs

All rats were observed in great detail at least once a day, during acclimatization and recovery.

The observations included at least: changes in the skin, fur, eyes and mucous membranes, in the respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral.

Body weight

The body weight of each rat was recorded during the week before of the start of treatment, twice weekly during the treatment weeks 1 to 13 and weekly afterwards, and before sacrifice.

Those rats subjected to the recovery period were weighed weekly. The mean body weights per group and sex were calculated twice a week from the individual weights.

Food intake

Before the beginning treatment, and then once a week during the treatment period and during the recovery period, the food intake per cage was recorded and the weekly mean intake per rat was calculated.

Ophthalmoscopy

These examinations included the cornea, crystalline lens, conjunctivae, sclera, iris and fundus. During each examination, the pupils were dilated. Examinations were taken before the beginning of the study and at the end.

Laboratory studies

Blood samples were extracted from the retro-orbital plexus from all animals. The animals were fasted in metabolism cages for approximately 16-18 hours before blood sampling but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Urine was also collected during the 16 to 18 hour fasting period.

SACRIFICE AND MACROSCOPIC EXAMINATION

At the end of the treatment all surviving rats were deprived from food for 18 hours before the sacrifice. Then they were deeply anaesthetized with sodium pentobarbital administered itraperitoneally and then exsanguinated by excision of the axillary vessels and aorta, except for those undergoing recovery, which were sacrificed at the end of that period.

Because the total number of animals exceeds the number that could be sacrificed in one day, the necropsies were carried out on several consecutive days. Tissue and organs samples were collected from all animals at necropsy and fixed in neutral phosphate buffered 4% formaldehyde solution.

HISTOLOGICAL EXAMINATION

Slides and all organs and tissues which were collected at terminal sacrifice from the animals of the control and high-dose groups were examined by the study pathologist.

STATISTICAL ANALYSIS

- The Dunnett-test (many to one t-test) based on a pooled variance estimate was applied for the comparison of the treated groups and the control groups for each sex when the variables were assumed to follow a normal distribution.
- The Steel-test (many-one rank test) was applied instead of the Dunnett-test when the data were not assumed to follow a normal distribution.
- Fisher's exact-test was applied to the ophthalmoscopy and macroscopic findings.
- Armitage/Cochran Trend Test was applied for non-neoplastic lesions, when appropriate.
- Student´s t-test was applied to some parameters analyzed during the recovery period.

RESULTS

- No mortality attributable to the treatment among the animals treated with the RCE.
- No noteworthy clinical signs were observed. Just during the treatment period, salivation was observed occasionally, after the administration in some animals of the animals at the three treatment groups (both sexes).
- Food intake among the animals treated with the RCE had no differences from the Control group during the treatment period neither during the recovery period.
- Among males, no statistically significant changes were observed about body weight gain. Among females, statistically significant differences were observed occasionally in body-weight gain among the females treated at 55 mg/kg on days 22, 64, 71, 75 and 82 of the treatment. The mean body weight and body-weight gain (%) in the course of the recovery period , both sexes, was similar to that observed in Control group.
- There were no ocular alterations related to the treatment.
- No changes of toxicological relevance were observed. Statistically significant differences with respect to the Control group were observed in some treated groups in a few parameters: higher mean corpuscular hemoglobin index (MCH), and lower reticulocyte count in males treated at 5 mg/kg, higher mean corpuscular volume (MCV) in males treated at 55 mg/kg and higher platelet values in females treated at 600 mg/kg. However, these differences were devoid of any toxicological significance. At the end of recovery period, no hematological differences were observed in the analyses performed.
- There were no gross lesions that could be attributed to test-item treatment. All findings were considered to be within the range of normal background lesions that may be seen in

rats of this strain and age. The necropsies carried out at the end of the recovery period revealed no noteworthy alterations.

- At the end of treatment period, no variations in organ weight were observed in any of the treatments groups of males. Among the females, the relative organ/weight of liver at the doses of 5 and 600 mg/kg was statistically higher to that recorded in the Control group.
- There were no gross lesions that could be attributed to treatment with test item. The microscopic examination revealed some findings that were considered to be within the range of normal background lesions that may be seen in rats of this strain and age.
- No changes were observed in blood biochemistry parameters in the analyses performed during week 13 of treatment. At the end of recovery period lower statistically significant differences with respect to the Control groups were observed in phosphorous and sodium values. Moreover these differences were fortuitous in nature.

CONCLUSION

- No noteworthy changes were observed after repeated oral administration of the test item, RCE, to rats for 13 weeks at dose levels of 5, 55 and 600 mg/kg.
- Few changes such lower body-weight gain, were observed occasionally in females treated at 55 mg/kg. Furthermore several changes in the hematological blood analyses were observed in all the treatment groups. However, these differences were devoid of any toxicological relevance.
- At the end of treatment period, some differences observed in females liver weight at the doses of 5 and 600 mg/kg could be attributed to the lower weight observed in Control females liver weight.
- No treatment-related histopathological alterations were observed at any of the administered doses.
- The changes observed in the hematological parameters at the end of treatment period are considered not of toxicological relevance and within the range of normal values.
- The changes in sodium and phosphorus levels observed at the end of recovery period were fortuitous in nature and considered within the range of variations recorded for these parameters. Furthermore no noteworthy alterations were observed in the necropsies performed.
- According to the results obtained, the NOAEL (No Observable Adverse Effect Level) may be established at 600 mg/Kg/day for the oral administration to rats during a period of 13 weeks.

XIII.2.6. ACUTE INTRAPERITONEAL TOXICITY IN RAT

The Acute Intraperitoneal Toxicity test on the RCE was carried out by the *Centro de Investigación y Desarrollo Aplicado, S.A.L.* (CIDASAL) in Barcelona, Spain. The study was performed following Good Laboratory Practices (GLP) according to the OECD Principles of Good Laboratory Practice and Directive 1999/11/EC.

The study was begun in March 2004 and the final report (No. CD04/9054T) was signed in June 2004. See Annex V for the complete study.

ANIMALS USED

26 rats Sprague Dawley "SD" (13 male and 13 female) were used in the study. The animals used in the Preliminary Study weighed between 125-153 g and those used in the Principal Study weighed 150–159 g and 135–149 g for the male and female rats, respectively.

The rats were housed in Makrolon cages (59.0 x 38.5 x 20.0 cm) in groups of maximum 6 rats of the same gender during the period of acclimatation. The cages were identified by means of a card indicating the number of the study and the housed animals, their sex, code of the tested product, dose, administration route, date of administration, date of entrance of the animals and person in charge of the study. The cages were distributed on the shelves in order to equilibrate any external factor (environmental conditions) that could have an influence on the animals.

The study was performed at a temperature of 20-25ºC, and a relative humidity of 30-65%.

The illumination was artificial and controlled to provide 12 hours of light (7:00 to 19:00 h) and 12 hours of darkness.

DIET

The animals had free access to a standard diet for rats SAFE A04C.

WATER

The animals had water *ad libitum* provided with bottles. The water is analyzed periodically to detect the presence of potential contaminants.

ROUTE OF ADMINISTRATION, FREQUENCY AND VOLUME

The test product, the RCE codified as IB0004, was administered intraperitoneally by means of a graduated syringe provided with a needle 25G (0.5 x 16 mm), 23G (0.6 x 25 mm) or 20 G (0.9 x 25 mm) depending on the concentration of the administered solution.

The administration was carried out in one time, with a volume of 10 ml/kg for the dose of 250 mg/kg and with a volume of 20 ml/kg for the rest of the doses. The injection lasted about 0.1 ml/second.

The volume of administration to each animal was determined according its weight at the moment of administration.

DOSE AND SIZE OF THE GROUPS

Preliminary Study

The RCE was administered as follows:

TREATMENT	DOSE (MG/KG)	No ASSIGNED TO THE ANIMALS	
		Male	Female
RCE (IB0004)	250	1, 2	3, 4
	500	5, 6	7, 8
	900	9, 10	11, 12
	1000	13, 14	15, 16

Principal Study

The RCE was administered as follows:

TREATMENT	DOSE (MG/KG)	No ASSIGNED TO THE ANIMALS	
		Male	Female
RCE (IB0004)	1000	17 to 21	22 to 26

OBSERVATIONS

Preliminary Study

After the administration, the rats were observed at least twice a day for 14 days. After this period, the animals were sacrificed.

Principal Study

During the day of the administration, the rats were observed frequently, in order to detect clinical signs. Additionally, the animals were observed at least twice a day for 14 days.

The observations included, at least, changes in the skin, hair, eyes and mucous membranes, in the respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

After this period of observation, the animals were sacrificed.

Corporal weight

All the rats were weighed before the administration and at half of the period of observation. Before their sacrifice, the animals were again weighed.

Sacrifice and *post-mortem* procedures

After the period of observation, all the animals were sacrificed by intraperitoneal injection of sodium pentobarbital, performing the necropsy of all the animals from the Principal Study.

The necropsy included the assessment of the intact animal and all of the superficial tissues, followed by the observation of the cranial, thoracic and abdominal cavities.

RESULTS

Preliminary Study

- None of the animals administered with the RCE at the doses of 250, 500, 900 and 1000 mg/kg died.

- Clinical signs in several animals were observed during the first days post administration: abnormal locomotion, hunched posture, piloerection, muscle relaxation and enlarged abdomen.
- The evolution of the corporal weight of all the animals was normal.

Principal Study

- None of the animals administered with RCE at a dose of 1000 mg/kg died.
- The animals showed several clinical signs along the period post treatment: abnormal locomotion, hunched posture, piloerection, muscle relaxation and enlarged abdomen. These clinical signs observed are related with the general appearance and activity of the animal and do not affect the functional capacity. These signs could be attributed to the administration of the formulation at a high volume (20 mL/kg/animal). From the 4th day post administration, no clinical signs were observed in the animals administered at the dose of 1000 mg/kg.
- The evolution of the corporal weight of all the animals was normal.
  White areas were observed in the hepatic surface of all the male rats and of four female rats. The fifth female rat did not show macroscopic alterations. One of the male rats presented dilated ileum. The white areas observed on the hepatic surface in some rats are considered as remains of the product due to that the administration route was intraperitoneal.

CONCLUSION

- According to the results obtained, the Minimum Lethal Dose of RCE is greater than 1000 mg/kg when administered intraperitoneally in rats Sprague Dawley.

XIII.3. OTHER ANIMAL STUDIES WITH THE ROOSTER COMBS EXTRACT

XIII.3.1. STUDY OF THE EFFECTS OF THE ROOSTER COMBS EXTRACT ON HYALURONIC ACID CONCENTRATION IN A HORSE MODEL

This double-blind randomized controlled and prospective clinical pilot trial, was performed at the "*Universitat Autònoma"* of Barcelona, Spain, during 2005-06.

The study was published in 2009 (Carmona *et al,* 2009). See Annex V for the complete study.

OBJECTIVE

To determine the effect of the oral administration of the RCE on safety and on clinical condition of horses with osteochondrosis (OCD).

EXPERIMENTAL DESIGN

12 horses aged between 8 and 36 months with a radiographic diagnose of OCD were randomly divided in two groups and assigned to receive orally 250 mg of the RCE or placebo during 60 days.

At the end of the treatment (day 60) and 30 days after finalization (day 90) a complete assessment of safety and clinical parameters were done.

RESULTS

Safety

- No adverse events related to the study products were reported.
- No significant changes were observed in plasma and synovial fluid analysis.

Efficacy

The results suggest that oral RCE administration increase HA concentration, which could be well related to improvements of the clinical condition of the affected joint.

XIII.4. HUMAN STUDIES

Human studies have also been performed on the RCE. Basically these are efficacy studies but with some evaluated safety parameters.

The safety of the RCE has been assessed in human studies. In two different studies (one in healthy adults and another in adults with osteoarthritis of the knee) no adverse effects have been reported as a result of the intake of the product at the recommended dose (80mg/d) during the studies periods.

XIII.4.1. ORAL ADMINISTRATION OF A YOGURT SUPPLEMENTED WITH A ROOSTER COMBS EXTRACT IN HEALTHY ADULTS

This randomised double-blind placebo-controlled nutrition intervention trial, evaluating the efficacy and safety of the RCE was performed in the Instituto POAL de Reumatología (Barcelona, Spain) between 2007 and 2008.

This study is actually under publication revision. See Annex V for the complete study.

OBJECTIVES

- To determine the safety of the product supplemented with the RCE taking into consideration the adverse reactions, physic and vital constants examination and general tolerance.
- To determine the effect derived from the daily consumption of a supplemented yogurt with a RCE on the joint function.

EXPERIMENTAL DESIGN

The study enrolled 40 adults, 2 groups of 20 persons. All subjects were given one yogurt daily. Depending on the treatment assigned, the yogurts were supplemented with 80 mg of the RCE or, the yogurts were not supplemented. Total duration of this study for each person was of 12 weeks. All subjects carried out four visits along the study.

Safety parameters

Hereafter, some of the efficacy and safety parameters used in this study:

1. Body weight changes
2. Blood pressure
3. Heart rhythm
4. Subjective adverse effects
5. Physical adverse effects

RESULTS

- No adverse events were reported during the study.
- No significant changes were observed in body weight, blood pressure and heart rhythm after eating non-supplemented or supplemented yoghurts
- Regarding efficacy of the product, which was the main objective of the study, oral supplementation with RCE improved joint mechanics and muscle function as determined through isokinetic testing, thus attenuating risk factors of OA.

XIII.4.2. EFFECT OF ROOSTER COMBS EXTRACT ON PAIN RELIEF AND QUALITY OF LIFE IN SUBJECTS WITH KNEE OSTEOARTHRITIS: A PILOT RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED TRIAL

This randomised double-blind placebo-controlled clinical trial, evaluating the efficacy and safety of the RCE compared to placebo, for the improvement of quality of life in adults with osteoarthritis of the knee, was performed at Miami Research Associates (Miami), US, in 2005.

The study was published in 2008 (Kalman *et al,* 2008). See Annex V for the complete study.

OBJECTIVES

- To determine the safety and tolerability of the RCE through the study.
- To determine the comparative differences between the RCE and placebo in quality of life and pain relief over 8 weeks.

EXPERIMENTAL DESIGN

The study enrolled 20 adults (both genders), age 40 and over, with a clinical diagnosis of osteoarthritis of the knee(s), but otherwise in good general health. Patients were given one capsule daily of the RCE after breakfast, at a dose of 80 mg/d, identical in appearance to active product. The randomisation ratio of the RCE to placebo was 1:1.

Efficacy and safety measures

Tolerability and safety parameters were the incidence and severity of adverse events reported throughout the study as well as changes in blood pressure, heart rate, and laboratory tests including complete blood cell count and biochemical profile. Treatment compliance was also recorded. Non-compliance was defined as taking less than 80% of the prescribed course of the study product. Use of rescue medication (paracetamol 500 mg) during the study period was also checked.

The primary efficacy variable was the comparative difference between the active and the placebo arms in scores of the pain subscale of WOMAC and bodily pain of SF-36v2.

The use of an unpaired Student t test to compare changes over time between treatment groups is algebraically equivalent to using a two-way linear model (ANOVA).

Analytical Populations

The *Safety population* consists of all 20 randomised subjects, 11 in the RCE group, and 9 in the placebo group.

The *Efficacy population* consists of 16 subjects, 8 in the RCE group, and 8 in the placebo group.

MAIN RESULTS

1. Safety Analysis

- No serious adverse events were reported during this study.
- Three adverse events were observed during the study period. One subject in the RCE group complained of acute non-target knee pain, unrelated to the study product, and voluntarily dropped out of the study. The two adverse events among placebo subjects, one diarrhea episode and one hypoesthesia of the tongue, were of mild intensity and were judged by the investigator as probably not related (diarrhea) and possibly related (hypoesthesia) to the study product. No significant changes were observed in vital signs, body weight, and results of laboratory tests.
- Overall, the RCE appeared to be well tolerated and safe within the study parameters (body weight or vital signs, and safety lab results).
- No alterations on biochemical profile or blood cell count were detected as a result of RCE supplementation.

2. Efficacy Analysis

Daily supplementation with RCE, 80 mg/day for 8 weeks, appeared to be effective in subjects with knee osteoarthritis for decreasing pain, improving physical function, and enhancing several aspects of quality of life.

XIII.5. TOXICITY OF SODIUM HYALURONATE

Published literature on toxicological studies shows that sodium hyaluronate is reasonably expected to be safe.

- Orl-Rat LD_{50} : > 800 mg/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

- Ipr-Rat LD_{50} : 1770 mg/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

- Scu-Rat LD_{50} : > 4 g/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

- Orl-Mus LD_{50} : 2400 mg/kg

  Drugs in Japan (Ethical Drugs), 849, 1990

- Scu-Mus LD_{50} : 4 g/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

- Scu-Dog LD_{50} : > 50 mg/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 19 (suppl 1), S13, 1991

- Orl-Rbt LD_{50} : > 1 g/kg

  Drugs in Japan (Ethical Drugs), 849, 1990

- Ipr-Rbt LD_{50} : 1820 mg/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

- Scu-Rbt LD_{50} : > 2 g/kg

  Yakuri to Chiryo. Pharmacology and Therapeutics, 12, 5369, 1984

Next abstract confirms the safety of sodium hyaluronate administered during 90 days in a subchronic toxicity study in rats. This study has special relevance due to that the sodium hyaluronate used was also obtained from an animal origin source (chicken) as our RCE.

- **Acute and subchronic oral toxicity in rats:**

Schauss AG, *et al*. Acute and subchronic oral toxicity studies in rats of a hydrolyzed chicken sternal cartilage preparation. *Food Chem. Toxicol*, 45(2):315-24, 2007:

Two acute and subchronic oral toxicity studies were conducted in rats to evaluate safety of a patented preparation of hydrolyzed chicken sternal cartilage (BioCell Collagen II) containing collagen type II, chondroitin sulfate, and hyaluronic acid. In the acute oral toxicity study, five males and five females of Sprague-Dawley rats were administered a single dose of 5000 mg of the test product per kg body weight and observed for 14 days. All animals survived and exhibited normal body weight gain throughout the study. Macroscopic necropsy examination conducted on day 15 revealed no gross pathological lesions in any of the animals. In the subchronic study, Sprague-Dawley rats (40 males, 40 females) were divided into four same-sex groups (10 animals/group). Animals in each group were administered daily either 0, 30, 300 or 1000 mg of the test product per kg of body weight for over 90 days. All animals survived and showed no significant changes in their body weights and histopathology. Although some differences were observed between the treated and control animals in several parameters, they were generally not dose-related or considered to be of toxicological significance. In conclusion, the results from the two oral toxicity studies with male and female young adult rats indicated that the test preparation from hydrolyzed chicken sternal cartilage collagen (BioCell Collagen II) was well tolerated at all four doses tested.

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D. Martinez-Puig, I. Möller, C. Fernández, C. Chetrit (2009) *Efficacy of Oral Administration of Yoghurt Supplemented with a Natural Extract Containing Hyaluronic Acid (Mobilee™) in Adults with Mild Joint Discomfort: A Randomized, Double-blind, Placebo-controlled Intervention Study*. Joint Bone Spine *(under revision*).

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