SCIENTIFIC OPINION

Scientific Opinion on Rooster Combs Extract[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to carry out the additional assessment for 'Rooster Combs Extract' (RCE) as a food ingredient in the context of Regulation (EC) No 258/97, taking into account the comments and objections of a scientific nature raised by Member States. Rooster combs extract results from a production process involving enzymatic hydrolysis of rooster combs and subsequent filtration, concentration and precipitation steps. The principle constituents of RCE are the glycosaminoglycans hyaluronic acid, chondroitin sulphate A and dermatan sulphate. The applicant intends to add RCE to a number of dairy products with a recommended maximum intake of 80 mg RCE per portion and per day. The target population is the general population, with the exception of pregnant women, children and people with adverse reactions to sodium hyaluronate and/or avian protein. In the high intake scenario for "consumers only", the highest daily intake would occur in adults in Belgium (0.788 g). The highest intake scenario for "all subjects" was estimated for adolescents in Denmark (0.427 g/day). The Panel notes that no adverse effects were observed at the highest tested dose of 600 mg/kg bw per day in a 90-day oral toxicity study in rats. Considering the nature, the natural occurrence and previous consumption of RCE constituents, the Panel is of the opinion that the margin between the intended as well as the estimated maximum possible intake of RCE in relation to the highest dose administered to rats without adverse effects in a subchronic oral toxicity study is sufficient. The Panel concludes that the novel food ingredient, Rooster Comb Extract, is safe under the proposed uses and use levels.

KEY WORDS

rooster Combs, glycosaminoglycans, hyaluronic acid, chondroitin sulphate, novel food, ingredient

[1] On request from the European Commission, Question No EFSA-Q-2012-00613, adopted on 31 May 2013.

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to carry out the additional assessment for 'Rooster Combs Extract' (RCE) as a food ingredient in the context of Regulation (EC) No 258/97, taking into account the comments and objections of a scientific nature raised by Member States.

Rooster combs extract results from a production process involving enzymatic hydrolysis of rooster combs and subsequent filtration, concentration and precipitation steps. The principle constituents of RCE are the glycosaminoglycans hyaluronic acid, chondroitin sulphate A and dermatan sulphate (chondroitin sulphate B). The Panel considers that the information provided on the manufacturing process as well as on the composition and specification of the novel food ingredient RCE is sufficient and does not raise safety concerns.

Rooster combs have a history of consumption in the European Union. Glycosaminoglycans, the main constituents of RCE, occur endogenously in mammalian organisms and are thus consumed as part of the normal diet.

The applicant intends to add RCE to liquid milk, milk-based products (milk-based fermented beverages), yoghurts and fromage frais with a recommended maximum intake of 80 mg RCE per portion and per day. The target population is the general population, with the exception of pregnant women, children, and people with adverse reactions to sodium hyaluronate and/or avian protein. Based on these proposed uses and use levels, the applicant conducted an intake estimation based on the Comprehensive European Food Consumption Database. Intake data were specified by country, food category, age group, for "all subjects" and for "consumers only". The mean daily intake estimates for "consumers only" and for "all subjects" were calculated to be highest for the Netherlands (0.455 g in the adult population) and for Denmark (0.255 g in adolescents), respectively. According to the model applied by the applicant for the high intake scenario for "consumers only", the highest daily intake would occur in adults in Belgium (0.788 g). The highest intake scenario for "all subjects" was estimated for adolescents in Denmark (0.427 g/day). The Panel notes that this type of intake methodology for fortified foods is generally considered to be "high intake", as a result of conservative assumptions made in the intake estimates where it is assumed that all food products within a food category contain the ingredient at the maximum specified level of use.

Rooster combs extract did not show mutagenic activity in tests for gene mutations in bacteria up to the highest tested dose of 5000 µg/plate in the presence and absence of metabolic activation. Considering the nature of the test material and the negative results in tests for gene mutations in bacteria, the Panel had no safety concerns related to genotoxicity. The Panel notes that no adverse effects were observed at the highest tested dose of 600 mg/kg bw per day in a 90-day subchronic oral toxicity study in rats.

The applicant provided two articles on two randomised placebo-controlled human studies. They included some endpoints on safety and tolerability but were primarily designed to study possible beneficial effects of RCE produced by the applicant. The Panel notes the low number of subjects, the relatively low dose, the number of safety endpoints studied, and the limited information regarding these safety endpoints (e.g. the parameters of the biochemical profile were not specified). The Panel considers that no conclusion can be drawn from the human studies on the safety of RCE. Panel considers that the risk of allergic reactions cannot be ruled out, but is not dissimilar to other products derived from chicken meat. The applicant's intended daily dose of 80 mg RCE corresponds to a production yield of one rooster comb, which corresponds to about 1 mg RCE per kg bw for an adult. A mean and high intake estimate of about 450 mg/day and 790 mg/day, respectively, for adults are derived from an intake assessment based on conservative assumptions. Considering the nature, the natural occurrence and previous consumption of RCE constituents, the Panel is of the opinion that the margin between the intended as well as the estimated maximum possible intake of RCE in relation to

the highest dose administered to rats without adverse effects in a subchronic oral toxicity study is sufficient.

The Panel concludes that the novel food ingredient, Rooster Comb Extract, is safe under the proposed uses and use levels.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On 8 February 2011, Bioiberica S.A. (Spain) submitted a request under Article 4 of the Novel Food Regulation (EC) N° 258/97 to place on the market 'Rooster Combs Extract' (RCE) as a novel food ingredient.

On 25 October 2011, the competent authorities of the United Kingdom forwarded to the Commission their initial assessment report, which came to the conclusion that RCE may be placed on the market.

On 10 November 2011, the Commission forwarded the initial assessment report to the other Member States. Several of the Member States submitted comments or raised objections.

The concerns of a scientific nature raised by the Member States can be summarized as follows:

- The mass balance according to the proposed specification indicates that some 20 % of the product has not been specified. The application itself indicates that, in addition to the 60-80 % sodium hyaluronate, the product contains glycosaminoglycans such as chondroitin sulphate and dermatan sulphate (about 20 %) and also partially hydrolysed proteins (about 20 %). The glycosaminoglycans were not, however, further determined either in the specification or in analyses of the product concerned.

- The cited nitrogen content (44 g/100 g) in the section "nutritional information" is many times higher than that specified in Tables 1-3 (not more than 8 % and mean of 6.43 %, respectively).

- The data on the nitrogen content are ambiguous, because the applicant's analyses do not distinguish between nitrogen molecules originating from protein and the N-acetyl-glucosamine component of hyaluronate (and other glycosaminoglycans). Hence, no reliable figures are available concerning the extract's maximum protein concentration either. Proteins are not specified separately, although the nitrogen content (≤ 8 %) is.

- The nitrogen mass balance according to total Kjeldahl nitrogen and the Lowry protein assay is inconclusive in itself: the mole percent of nitrogen in sodium hyaluronate is some 3.5 %, which, with an average content of 65.5 % sodium hyaluronate, would mean a nitrogen content of some 2.3 %. If we add the 0.8 % protein nitrogen (ca. 16 % of the protein content of 5.1 %), the total nitrogen content is 3.1 %. On average, however, a total nitrogen content of 6.5 % was found. Even an additional examination of the nitrogen content in the glycosaminoglycans does not adequately clarify the nitrogen distribution in the product.

- The specification of the product concerned differs significantly from that of the European Pharmacopoeia for sodium hyaluronate, which, for example, indicates a maximum sulphated glycosaminoglycan content of 1 % and a maximum protein content of 0.3-0.1 %.

- No tests confirming the identity of the main constituents and other extraneous substances have been conducted or presented.

- It is not clear how the fibre content (36 g/100 g) has been determined.

- The application does not identify the test facility that analysed the product. It should be noted that only test laboratories accredited for the tests in question should be used for analyses. Also no test reports are available.

- The information provided regarding the production process was considered to be insufficiently detailed to assess the applicant's assertion that the substances making up the extract are roughly 1 % of those making up the rooster combs used as source material.

- It is necessary to better specify the methods for adding the NF to the milk products they are intended for. The impact of the production process for these products such as, for example, the stability of the NF under heat treatments ought to be studied.

- The applicant claims that during the production process the product undergoes a heat treatment which inactivates the production enzyme. However, the relevant process conditions are not specified and the summary data do not adequately demonstrate the effectiveness of the inactivation procedure. Moreover, it is not apparent from the available information whether the inactivated enzyme is removed. Consequently, it is not possible to tell what proportion of the total protein content is accounted for by the enzyme. A Member State also asked for assurance that the enzyme used is of food-grade.

- Previous consumption data would be useful.

- Without a clear specification of the use levels in the relevant products referred to by the applicant, it is not possible to reliably estimate intake of the novel product.

- The petitioner must justify the limits that it has proposed for contaminants.

- The absence of data on the metabolism of the NF was regretted.

- The applicant claims to have used the product which is the subject of the application in the analyses. The documentation does not indicate, however, whether the test substances "Hyal-Joint" and "IB 0004" meet the specification of the product concerned or differ from it.

- The applicant has submitted only a draft report on the 90-day study with rats. On the basis of the pathology information provided, the study appears to have been comprehensive, but it is not clear why OECD guideline 452 for chronic research has been used in this context. In view of the paucity of safety data, a signed finalised version of this essential study report is a prerequisite for making a definitive assessment.

- The allergenicity should be addressed. In the context of possible allergenicity, the size distribution of the protein hydrolysis fragments is important as well.

- The additional data provided by the applicant does not conclusively demonstrate the absence of serological cross-reactivity between anti-chicken egg protein IgE and proteins in the extract, because the size and characteristics of the serum pool of patients with egg protein allergy was not clearly stated.

- The safety of the NF in young people has not been adequately studied.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Food Safety Authority is asked to carry out the additional assessment of 'Rooster Combs Extract' as a food ingredient in the context of Regulation (EC) No 258/97.

EFSA is asked to carry out the additional assessment and to consider the elements of scientific nature in the comments raised by the other Member States.

ASSESSMENT

In accordance with Commission Recommendation 97/618/EC, 'Rooster Combs Extract (RCE)' is allocated to Class 2.1 'a complex (non-GM derived) novel food ingredient, the source of the novel food having a history of food use in the community'. The assessment of the safety of this novel food ingredient is based on data supplied in the original application, the initial assessment by the competent authority of the United Kingdom, the comments and objections of the other Member States, and the responses of the applicant to these questions and those of the United Kingdom. The data are required to comply with the information required for novel foods of Class 2.1, i.e. structured schemes I, II, III, IX, X, XI, XII and XIII. It is noted that the novel food ingredient (NFI) is intended by the applicant to be added to certain dairy products (i.e. milk-based fermented beverages, yoghurts, milks and fromage frais) to support joint health of the general population. This assessment concerns only risk that might be associated with consumption, and is not an assessment of the efficacy of 'RCE' with regard to any claimed benefit.

1. Specification of the Novel Food (NF)

Rooster combs extract (RCE) results from a production process involving enzymatic hydrolysis of rooster combs and subsequent filtration, concentration and precipitation steps. The principle constituents of RCE are the glycosaminoglycans hyaluronic acid, chondroitin sulphate A and dermatan sulphate (chondroitin sulphate B). Their structures are depicted in Figure 1.

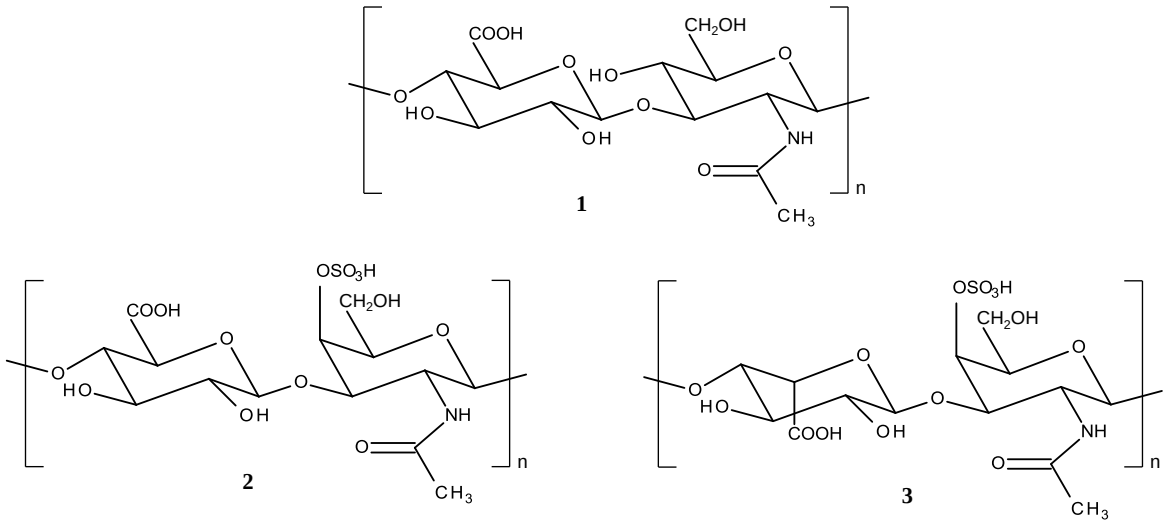

Figure 1: Structures of hyaluronic acid (**1**), chondroitin sulphate A (**2**), and dermatan sulphate (chondroitin sulphate B) (**3**)

Hyaluronic acid (1) is a non-sulphated glycosaminoglycan. Its basic unit is a disaccharide of D-glucuronic acid and N-acetyl-D-glucosamine linked by a ß-1,3-glycosidic bond; these disaccharides are polymerized via ß-1,4-glycosidic bonds.

Chondroitin sulphate A (2) is a C4-sulphated glycosaminoglycan. Its basic units are disaccharides of D-glucuronic acid and N-acetyl-D-galactosamine linked by a ß-1,3-glycosidic bond; these are

polymerized via ß-1,4-glycosidic bonds. In the disaccharide unit of dermatan sulphate (chondroitin sulphate B) (3) glucuronic acid is replaced by the epimeric iduronic acid.

The applicant provided compositional data on ten batches of RCE produced in 2008 and 2009 (Table 1).

Table 1: Compositional data of RCE batches produced in 2008 and 2009

Parameter	8/0001 Feb 08	8/0010 Apr 08	8/0021 Jul 08	8/0038 Nov 08	9/0001 Jan 09	9/0008 Feb 09	9/0009 Mar 09	9/0010 Mar 09	9/0011 Apr 09	9/0012 Apr 09
Glucuronic acid, expressed as sodium hyaluronate (%)	65	61	65	68	67.5	69	65	64	62	67
pH	7.2	7.4	6.7	6.3	6.0	6.6	6.2	6.4	6.5	6.7
Chlorides (%)	0.5	0.7	0.4	0.4	0.4	0.5	0.6	0.8	0.5	0.5
Nitrogen (Kjeldahl) (%)	6.0	5.0	7.0	7.0	7.0	7.0	7.0	6.0	6.0	6.0
Loss on drying (%)	8.3	7.7	5.5	5.0	5.4	6.0	5.0	6.5	6.0	6.0
Heavy metals (mg/kg)	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10	≤ 10
Mercury (mg/kg)	< 0.10	-	-	< 0.10	-	-	-	-	-	< 0.10
Arsenic (mg/kg)	< 1	-	-	< 1	-	-	-	-	-	< 1
Cadmium (mg/kg)	< 1	-	-	< 1	-	-	-	-	-	< 1
Chromium (mg/kg)	< 10	-	-	< 10	-	-	-	-	-	< 10
Lead (mg/kg)	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Dioxins/Furans (pg/g)	0.024	-	-	0.04	-	-	-	-	-	0.07
PCBs (pg/g)	0.004	-	-	0.006	-	-	-	-	-	0.01
Total aerobic count (cfu/g)	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$	$\leq 10^2$
E. coli per g	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Salmonella per g	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S. aureus per g	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P. aeruginosa per g	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

'n.d.' = not detected; '-' = not tested

In addition, data were reported on the contents of glucuronic acid, expressed as sodium hyaluronate for a total of 45 batches of RCE produced in 2008 (mean: 64.6 %; median: 65.0 %; standard deviation: 2.57; standard error: 0.41).

Upon request by the Panel, the applicant provided more refined compositional data, based in particular on specific analyses of hyaluronic acid, chondroitin sulphate and dermatan sulphate, using methods based on capillary electrophoresis and HPLC. In addition, revised data for the contents of fibre, free amino acids and protein were provided. The data given for four batches of RCE manufactured between September 2011 and November 2012, and for the batch (FTAH04-08, "Hyal Joint") used for the subchronic study and the genotoxicity tests, are shown in Table 2.

Table 2: Compositional data of industrially produced batches of RCE

Parameters (%)	Batches					Method
	12/0001	12/0015	12/0027	12/0040	FTAH04-08*	
Hyaluronic acid	61.7	63.5	65.6	69.1	62.1	HPLC (Coleman et al., 1997) and capillary electrophoresis (Malavaki et al., 2008)
Chondroitin sulphate A	2.13	2.21	2.23	2.43	0.45	Capillary electrophoresis (Malavaki et al., 2008)
Chondroitin sulphate B	11.27	12.23	17.12	8.85	11.48	Capillary electrophoresis (Malavaki et al., 2008)
Fibre	0.4	< 0.1	< 0.1	< 0.1	< 0.2	Weende
Free amino acids	1.7	1.3	1.4	1.4	0.3	HPLC
Protein	21.4	18.5	15.5	18.1	12.6	Lowry
Loss on drying	6	7	6	8	8	Eur. Ph. 2.2.32

* used for genotoxicity and subchronic toxicity testing

In response to the objections by Member States and the request by the Panel, the applicant also provided data on the protein contents determined in several batches of RCE using the Lowry method. The protein contents in batches of RCE determined by the Lowry method ranged between 13.0 (batch No.08/0021) and 21.4 % (batch No. 12/0015).

The applicant proposed the specifications shown in Table 3.

Table 3: Specifications for RCE as proposed by the applicant

Parameter	Limits	Method
Glucuronic acid, expressed as sodium hyaluronate (%)	60 - 80	Eur. Ph. Monograph 1472
Hyaluronic acid (%)	60 - 80	HPLC (Coleman et al., 1997) and capillary electrophoresis (Malavaki et al., 2008)
Chondroitin sulphate A (%)	≤ 5	Capillary electrophoresis (Malavaki et al., 2008)
Dermatan sulphate (Chondroitin sulphate B) (%)	≤ 25	Capillary electrophoresis (Malavaki et al., 2008)
Appearance	White or almost white hygroscopic powder	visual
pH	5.0 - 8.5	Eur. Ph. 2.2.3
Chlorides (%)	≤ 1	Mohr
Nitrogen (Kjeldahl) (%)	≤ 8	Eur. Ph. 2.5.9
Protein (Lowry) (%)	≤ 25	Eur. Ph. 2.5.33
Loss on drying (%)	≤ 10	Eur. Ph. 2.2.32
Heavy metals (mg/kg)	≤ 10	USP 231
Mercury (mg/kg)	≤ 0.1	Eur. Ph. 2.2.58
Arsenic (mg/kg)	≤ 1	Eur. Ph. 2.2.58

Parameter	Limits	Method
Cadmium (mg/kg)	≤ 1	Eur. Ph. 2.2.58
Chromium (mg/kg)	≤ 10	Eur. Ph. 2.2.58
Lead (mg/kg)	≤ 0.5	Eur. Ph. 2.2.58
Dioxins and furans (pg/g)	≤ 2.0	EPA Method 1613
PCBs (pg/g)	≤ 4.0	EPA Method 613
Microbiological parameters		
Total viable aerobic count	≤ 10^2 cfu/g	Eur. Ph. 2.6.12
Escherichia coli	absence/1g	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

The Panel considers that the information provided on the composition, specification and data from batch testing are sufficient and do not raise safety concerns.

2. Effect of the production process applied to the NF

The production process of RCE includes an enzymatic hydrolysis, followed by filtration, concentration and precipitation steps.

A flowchart of the production process including a mass balance for each manufacturing step has been provided; starting from an initial loading of 5 000 kg of rooster combs, 50 kg of RCE is obtained as final product.

According to the applicant, rooster combs are sourced from poultry declared fit for human consumption in authorised slaughterhouses.

An alcalase is used for the enzymatic hydrolysis. The commercially available enzyme preparation employed is produced from a non-genetically modified strain of *Bacillus licheniformis*. The supplier provided a statement on the current approval status referring to the acceptance by the Danish Food Authority for it to be used for production of protein hydrolysates, and to the inclusion in the French positive list regarding the use of processing aids in the manufacturing of human food.

After the hydrolysis step, the enzyme is inactivated by heat-treatment. The inactivation was demonstrated by determining enzyme concentrations and activities in three batches before and after the heat-treatment. Before the thermal treatment, the enzyme protein concentration determined via ELISA ranged from 7.63 x 10^5 to 8.24 x 10^5 ng/g. After the inactivation step, the concentration was below the limit of detection of the employed assay (102 ng/g). The enzyme activity (4.35-4.70 x 10^{-2} AU/g before the heat treatment) was also below the limit of detection of the employed assay (5.8 x 10^{-6} AU/g) after the heat-treatment.

According to a certificate of the supplier, the salt (NaCl) used in the precipitation step is of food grade. The NaCl concentration is adjusted. The concentration of chlorides in the final product is less than 1 %.

The applicant states that its quality management system is based on ISO 9001 standards, which include a hazard analysis and critical control points system (HACCP).

Studies under accelerated storage conditions (40 ± 2º C / 75 ± 5 % Relative Humidity, RH, for 6 months) and long-term storage conditions (25 ± 2º C / 60 ± 5 % RH, 40-43 months) have been

conducted with three different production batches of RCE. The Panel considers that storage under the employed conditions, using as a primary packaging a triple low density polyethylene bag, and a metal drum as a secondary packaging, does not affect the stability of the RCE.

The stability of six different concentrations (from 0.16-1.28 mg RCE/g) in yoghurts was assessed under refrigerated storage conditions for 1 and 1.5 months, which covers the mean shelf life of a standard commercial yoghurt (normally three weeks). Analyses show that RCE remained stable with only minor variations in RCE concentration, which are considered not relevant for the safety of the products.

The production process encompasses steps commonly used in food technology. The Panel considers that the production process is sufficiently described by the applicant and does not raise safety concerns.

3. History of the organism used as a source

Rooster Combs Extract is obtained from rooster combs from *Gallus gallus*. 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 in length and weigh more than 8 grams. Rooster combs have a history of human consumption in Europe and continue to be part of the normal diet in some countries, including frequently consumed dishes such as home-made recipes (stews) and industrially prepared soup concentrates.

4. Anticipated intake/extent of the use of the NF

The applicant intends to add RCE to liquid milk, milk-based products (milk based fermented beverages), yoghurts and fromage frais with a recommended maximum intake of 80 mg RCE per portion and per day. The target population is the general population, with the exception of pregnant women, children and people with adverse reactions to sodium hyaluronate and/or avian protein.

The maximum concentrations intended for each of the food categories are given in Table 4.

Table 4: Intended maximum concentrations of RCE

Dairy product	Maximum use level (mg/portion)	Portion size [a] (g)	Maximum level (mg/100g food)
Liquid milk	80	200	40
Milk-based product (milk based fermented beverages)	80	100	80
Yoghurt	80	125	64
Fromage frais	80	75 [b]	106.7

[a] Portion sizes according to those established by the UK Food Standards Agency (http://www.food.gov.uk/multimedia/pdfs/ann3portions.pdf)
[b] Average size of retail products in Spain 2012

The applicant indicated the use of the Comprehensive European Food Consumption Database (EFSA, 2011) for the intake estimates. These data were specified by country, food category, age group, for "all subjects" and for "consumers only". The mean intake estimate was calculated by adding the sum of the mean intake from all four food categories (Table 5, Appendix). The estimated high intake scenario of the applicant was based on the sum of the mean intake values of three food matrices (milk, yoghurt and fromage frais) plus the highest 95th percentile intake estimated for the four food

categories, which was estimated for milk-based products. Since "Fromage frais" is not a category in the Comprehensive European Food Consumption Database, the applicant used total cheese consumption as the starting point. Based on Spain and UK survey data, the percentage of fresh cheese consumption from total cheese intake has been calculated for Spain (27 %) and the UK (14 %). The figure for Spain (27 %) was then used as a proxy of fresh cheese intake for other European countries.

The mean daily intake estimates for "consumers only" and for "all subjects" were calculated to be highest for the Netherlands (0.455 g in the adult population) and for Denmark (0.255 g in adolescents), respectively (Table 5, Appendix).

According to the model applied by the applicant for the high intake scenario for "consumers only", the highest daily intake would occur in adults in Belgium (0.788 g). The highest intake scenario for "all subjects" was estimated for adolescents in Denmark (0.427 g/day) (Table 6, Appendix).

The Panel notes that this type of intake methodology for fortified foods is generally considered to be "high intake", as a result of conservative assumptions made in the consumption estimates where it is assumed that all food products within a food category contain the ingredient at the maximum specified level of use.

5. Information from previous exposure to the NF or its source

The applicant notes that rooster combs have been consumed in the EU. Also, there are several food supplements on the EU market (Belgium, France, Germany, Ireland, Italy, Portugal, Spain and UK), containing sodium hyaluronate. According to the applicant, these supplements do not specify the source of sodium hyaluronate except one which is obtained by microbial fermentation.

Rooster Combs Extract's compounds are present in a comb at an approximate proportion of 1 %. The applicant's intended daily dose of 80 mg represents the production yield from a single rooster comb.

Glycosaminoglycans, the main constituents of RCE, occur endogenously in mammalian organisms and are thus consumed as part of the normal diet.

6. Nutritional information on the NF

The applicant states that RCE in dairy products is not intended to replace any existing food ingredient. Based on the information provided on the nature, composition and proposed use levels, the Panel considers that the intake of RCE is not nutritionally disadvantageous.

7. Microbiological information on the NF

The applicant has provided microbiological specifications and has also supplied results of analyses for ten independent lots of RCE. All batches comply with the specifications.

The Panel has no safety concerns with regard to the microbiological specifications of the novel food ingredient.

8. Toxicological information on the NF

8.1. Absorption, distribution, metabolism and elimination (ADME)

On request of the Panel to provide information on the absorption, distribution, metabolism and excretion (ADME) of hyaluronic acid (HA), which is the main constituent of RCE, the applicant provided the results of a study using the *ex vivo* everted gut sac model. The Panel notes that the

applicant did not provide data on hyaluronic acid, but determined the amount of glycosaminoglycans passing through the intestinal wall by applying a spectrophotometric method, which detects sulphated glycosaminoglycans (Farndale et al., 1982). The authors concluded that absorption of RCE through the duodenum, jejunum and ileum was 38 %, 22 % and 8 %, respectively (Torrent et al., 2010).

The applicant also provided information on the metabolic fate of HA obtained from the scientific literature. After oral administration of a single dose of commercial food grade sodium hyaluronate preparations (labelled with ^{99m}Tc) to rats, total excretion of the ingested dose over 72 h was 84.6-92.3 % in faeces and 2.0-3.2 % in urine. Radioactivity was detected at different time points in blood and all organs examined, except for the brain, with organs of the gastrointestinal tract containing the vast majority. Based on the total results of this study, which also included data from dogs, the authors estimated that the percentage of HA entering the systemic circulation after oral administration is similar to that reported for other glycosaminoglycans, i.e. 5-20 % (Balogh et al., 2008). This conclusion is not in line with that of other investigators, who compared the distribution profiles and elimination pathways of ^{99m}Tc-labelled and ^{14}C-labelled HA, and found only negligible radioactivity absorption from the gastrointestinal tract after oral administration (Laznicek et al., 2012).

The Panel notes that the information provided on ADME is limited and inconsistent and does not allow conclusions to be drawn.

8.2. Toxicological studies

The applicant has conducted a range of toxicological studies, which are described below. The relevant results from these studies are summarised in the publication of Canut et al. (2012). On request from EFSA, the applicant provided compositional data showing that the test material used in the studies on subchronic toxicity and genotoxicity (designated 'Hyal-Joint') complied with the proposed specification for RCE (see section 1, Table 3).

8.2.1. Genotoxicity

Tests for gene mutations in bacteria were performed according to OECD Guideline 471, and in compliance with GLP principles (Bioiberica, 2008). Using *Salmonella typhimurium* strains TA 1 535, TA 1 537, TA 98 and TA 100, and the *Escherichia coli* strain WP2 *uvr*A pkM101, RCE did not show mutagenic activity up to the highest tested dose of 5000 µg/plate in the presence and absence of metabolic activation (S9 mix). Considering the nature of the test material and the negative results in tests for gene mutations in bacteria, the Panel had no safety concerns related to genotoxicity.

8.2.2. Acute toxicity

In an acute oral toxicity study (GLP complying) RCE was administered via stomach tube to Sprague Dawley rats (Bioiberica, 2004). There were no indications of adverse effects up to the highest tested dose of 2 000 mg/kg body weight (bw).

8.2.3. Subacute toxicity

A two-week dose range finding study (GLP complying) was performed in Wistar rats (Bioiberica, 2005). Rooster Comb Extract was administered by gavage to groups of 5 male and 5 female animals (strain HsdBrlHan:WIST) at doses of 200, 400 or 600 mg/kg bw per day for two weeks. The control group just received the vehicle (distilled water). Based on the results of this study a dose of 600 mg/kg bw per day was considered as an appropriate high dose in a 4-week study.

In a 4-week study (subacute toxicity study), groups of 10 male and 10 female rats of the same strain received RCE at doses of 0, 5, 55 or 600 mg/kg bw per day via stomach tube. According to the study

report, no adverse effects were observed at the highest dose tested. The Panel notes that the study report (available only in Spanish) was not signed, did not contain a GLP certificate, and did not make reference to OECD Guideline 407.

8.2.4. Subchronic toxicity

Rooster Comb Extract was also tested in a subchronic oral toxicity study, which was performed according to Commission Directive 96/54/EEC and in compliance with GLP principles (Bioiberica, 2009). Groups of 10 male and 10 female Wistar rats (strain HanBrl:Wist) were administered doses of 5, 55 or 600 mg/kg bw per day via stomach tube for 13 weeks. The control group received the vehicle alone. Two additional groups of 5 male and 5 female animals were administered the high dose or vehicle for 13 weeks and received a standard rodent diet for 4 weeks thereafter (recovery period).

One female animal of the control group died on day 5 of the treatment period because of incorrect gavage. Regular observations of the remaining animals revealed no clinically relevant findings. Salivation was recorded occasionally after administration in some animals of the three treatment groups. Body weight development and food consumption were comparable in all groups. Haematology analysis showed isolated statistically significant differences in relation to the control group, which are regarded as incidental findings (i.e. higher mean cellular haemoglobin (MCH) in males of the low dose group; higher mean cellular volume (MCV) and lower mean reticulocyte counts in males of the intermediate dose group). Females of the high dose group showed significantly higher mean platelet counts at the end of the treatment period, but no difference was noted at the end of the 4-week recovery period. Taking into account the absence of changes in related parameters as well as the historical control data provided by the applicant upon request, the Panel does not consider the observed difference as a treatment-related effect. No significant differences were noted in clinical-chemistry and urine analyses.

Determination of the weights of selected organs and tissues at necropsy after 13 weeks showed significantly higher liver weights in females of the low dose group (absolute and in relation to brain weight) and the high dose group (absolute and in relation to body weight and brain weight). These changes were not dose-related, and at the end of the recovery period female liver weights in the high dose group were significantly lower compared with the controls. Histopathological examinations of the liver as well as other selected organs and tissues did not reveal relevant differences in the types and incidences of findings between the high dose and the control group. The Panel notes that no adverse effects were observed at the highest dose tested of 600 mg/kg bw per day.

8.3. Allergenicity

The applicant proposes labelling to inform people who are allergic to avian proteins. The Panel considers that the risk of allergic reactions cannot be ruled out, but is not dissimilar to other products derived from chicken meat.

8.4. Human studies

The applicant provided two articles on two randomised placebo-controlled human studies. They included some endpoints on safety and tolerability but were primarily designed to study possible beneficial effects of RCE produced by the applicant. In the study by Kalman et al. (2008), 20 adults with osteoarthritis of knee(s) but otherwise of good general health received one capsule per day with 80 mg of RCE or placebo after breakfast over 8 weeks. Tolerability and safety parameters were the incidence and severity of adverse events reported throughout the study as well as changes in blood pressure and heart rate. Laboratory tests included 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. No serious adverse events were reported. Two adverse events

were recorded for the placebo group, one subject of the RCE group complained of acute knee pain. No significant changes were observed in vital signs, body weight or laboratory tests between the test and the control group. The Panel notes that the article does not specify which biochemical endpoints were tested. The Panel considers that the low number of subjects, the relatively low dose, the number of safety endpoints studied, and the limited information on these safety endpoints (e.g. the parameters of the biochemical profile were not specified) do not allow conclusions to be drawn on the safety of RCE. The other RCT enrolled 40 adults who were given one 80 mg RCE-supplemented or non-supplemented yoghurt per day for 12 weeks (Martinez-Puig et al., 2012). While the article indicated that only blood pressure, heart rate and body weight changes were studied safety endpoints, the applicant also claims that "subjective and physical adverse events" were endpoints. No significant differences were reported between the two groups. The Panel notes that the relatively low dose, the number of safety endpoints studied, and the limited information on these safety endpoints do not allow conclusions to be drawn on the safety of RCE. The Panel considers that no conclusion can be drawn from the human studies on the safety of RCE.

DISCUSSION

The Panel considers that the information provided on the manufacturing process as well as on the composition and specification of the novel food ingredient RCE is sufficient and does not raise safety concerns.

Rooster combs have a history of consumption in the European Union. Glycosaminoglycans, the main constituents of RCE, occur endogenously in mammalian organisms and are thus consumed as part of the normal diet.

The applicant's intended daily dose of 80 mg RCE corresponds to a production yield of one rooster comb which corresponds to about 1 mg RCE per kg bw for an adult. A mean and high intake estimate of about 450 mg/day and 790 mg/day, respectively, for adults are derived from an intake assessment based on conservative assumptions. Considering the nature, the natural occurrence and previous consumption of RCE constituents, the Panel is of the opinion that the margin between the intended as well as the estimated high percentile intake of RCE in relation to the highest dose administered to rats without adverse effects in a subchronic oral toxicity study is sufficient.

CONCLUSION

The Panel concludes that the novel food ingredient, Rooster Comb Extract, is safe under the proposed uses and use levels.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier on 'rooster comb extract'. 9 June 2012. Submitted by Bioiberica A.S. Additional information was submitted on 9 January 2012 and on 21 February 2013.

2. Letter from the European Commission to the European Food Safety Authority with the request for an opinion on the safety of 'rooster comb extract'. SANCO E6/AK/bs, Ref. Ares (2012)610804 – 22/05/2012.

3. Initial assessment report carried out by the United Kingdom, 'rooster comb extract' as novel food ingredients, Initial assessment under Article 4 of Regulation (EC) No 258/97".

4. Member States' comments and objections.

5. Response by the applicant to the initial assessment report and the Member States' comments and objections.

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ABBREVIATIONS

Bw	Bodyweight
NF(I)	Novel Food (Ingredient)
NOAEL	No observed-adverse-effect level
RCE	Rooster comb extract

APPENDIX

Table 5: Mean intake estimate based on the sum of the estimated mean intake from the four food categories

POPULATION CHARACTERISTICS				MILK + RCE		MILK BASED PRODUCTS + RCE		YOGHURT + RCE		FROMAGE FRAIS + RCE		ADDITION OF THE FOUR FOODS	
				Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects
	Age Group	Total No of Subjects	% Consumers	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)
BELGIUM	11-18	411	70.4	0.082	0.058	0.104	0.006	0.062	0.014	0.046	0.034	**0.294**	**0.112**
	19-64	820	62.9	0.049	0.03	0.191	0.004	0.069	0.025	0.058	0.046	**0.367**	**0.105**
	>65	303	58.5	0.034	0.02	0.115	0.001	0.083	0.028	0.054	0.043	**0.286**	**0.092**
CYPRUS	19-64	264	87.1	0.085	0.074	0.121	0.002	0.019	0.007	0.039	0.032	**0.264**	**0.115**
CZECH REP.	11-18	288	96.6	0.072	0.07	0.095	0.003	0.081	0.038	0.039	0.030	**0.287**	**0.141**
	19-64	1484	89.1	0.041	0.036	0.124	-	0.081	0.027	0.048	0.033	**0.294**	**0.096**
DENMARK	11-18	478	99.8	0.136	0.136	0.102	0.057	0.046	0.034	0.029	0.028	**0.313**	**0.255**
	19-64	2816	99.8	0.102	0.101	0.073	0.023	0.045	0.03	0.033	0.033	**0.253**	**0.187**
	>65	309	100	0.092	0.092	0.062	0.011	0.06	0.037	0.029	0.028	**0.243**	**0.168**
FINLAND	19-64	1432	90.9	0.125	0.114	-*	-*	0.127	0.073	0.048	0.039	**0.300**	**0.226**
	>65	432	93.3	0.124	0.116	-*	-*	0.139	0.073	0.034	0.025	**0.297**	**0.214**
FRANCE	11-18	844	86.7	0.074	0.064	0.08	0.003	0.047	0.035	0.038	0.037	**0.239**	**0.139**
	19-64	1599	70.3	0.047	0.034	0.051	0.001	0.059	0.041	0.052	0.050	**0.209**	**0.126**
	>65	167	63.3	0.042	0.026	-	-	0.054	0.033	0.057	0.056	**0.153**	**0.115**
GERMANY	11-18	602	59.6	0.084	0.049	0.154	0.005	0.065	0.017	0.040	0.030	**0.343**	**0.101**

	19-64	6011	57.7	0.062	0.035	0.155	0.002	0.081	0.029	0.049	0.039	**0.347**	**0.105**
	>65	989	49.3	0.054	0.026	0.128	-	0.080	0.033	0.047	0.04	**0.309**	**0.099**
HUNGARY	19-64	956	89.0	0.063	0.056	0.131	0.034	0.063	0.021	0.035	0.026	**0.292**	**0.137**
	>65	192	93.2	0.063	0.059	0.14	0.042	0.062	0.022	0.036	0.026	**0.301**	**0.149**
IRELAND	19-64	949	99.1	0.107	0.106	0.229	-	0.029	0.01	0.021	0.018	**0.386**	**0.134**
ITALY	11-18	210	85.0	0.071	0.06	-*	-*	0.052	0.011	0.065	0.063	**0.188**	**0.134**
	19-64	1742	75.3	0.054	0.041	-*	-*	0.056	0.014	0.066	0.064	**0.176**	**0.119**
	>65	224	77.2	0.057	0.044	-*	-*	0.052	0.01	0.058	0.056	**0.167**	**0.110**
LATVIA	11-18	231	49.2	0.055	0.027	0.118	0.001	0.082	0.033	0.036	0.022	**0.291**	**0.083**
	19-64	483	37.0	0.045	0.017	0.138	-	0.096	0.032	0.051	0.030	**0.330**	**0.079**
THE NETHERLAND	19-64	481	64.1	0.111	0.071	0.159	0.023	0.136	0.067	0.049	0.040	**0.455**	**0.201**
SPAIN	11-18	612	94.0	0.132	0.124	0.128	0.018	0.091	0.066	0.029	0.017	**0.380**	**0.225**
	19-64	939	95.7	0.123	0.117	0.072	0.002	0.061	0.036	0.031	0.023	**0.287**	**0.178**
SWEDEN	11 -18	952	93.5	0.133	0.124	0.11	0.04	0.08	0.041	0.019	0.012	**0.342**	**0.217**
	19-64	1041	86.0	0.109	0.094	0.043	-	0.094	0.058	0.033	0.031	**0.279**	**0.183**
UNITED KINGDOM	19-64	1673	97.0	0.086	0.084	0.034	0.003	0.033	0.014	0.025	0.022	**0.178**	**0.123**

* The applicant did not provide figures for the respective food categories for Italy and Finland. On request to EFSA, the applicant responded that the food categories available in the database did not match with the food products intended by the applicant. The figures for the intake estimate fro all food categories are therefore an underestimate for the two countries.

Table 6: High intake scenario (sum of estimated mean intake from three food categories plus the highest 95th percentile intake which was estimated for "milk based products")

POPULATION CHARACTERISTICS				MILK + RCE		YOGHURT + RCE		FROMAGE FRAIS + RCE		MILK BASED PRODUCTS Percentile 95th		ADDITION OF THE THREE FOODS (milk. milk-based. yoghurts)		ADDITION OF THE THREE FOODS + MILK BASED PRODUCTS Percentile 95th	
				Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects	Consumers only	All subjects
	Age Group	Total No of Subjects	% Consumers	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)	Mean (g)
BELGIUM	11-18	411	70.4	0.082	0.058	0.062	0.014	0.046	0.034	0.219	0.052	0.19	0.106	**0.409**	**0.158**
	19-64	820	62.9	0.049	0.03	0.069	0.025	0.058	0.046	0.612	-	0.176	0.101	**0.788**	**0.101**
	>65	303	58.5	0.034	0.02	0.083	0.028	0.054	0.043	0.206	-	0.171	0.091	**0.377**	**0.091**
CYPRUS	19-64	264	87.1	0.085	0.074	0.019	0.007	0.039	0.032	0.299	-	0.143	0.113	**0.442**	**0.113**
CZECH REP.	11-18	288	96.6	0.072	0.07	0.081	0.038	0.039	0.03	0.12	-	0.192	0.138	**0.312**	**0.138**
	19-64	1484	89.1	0.041	0.036	0.081	0.027	0.048	0.033	0.2	-	0.17	0.096	**0.370**	**0.096**
DENMARK	11-18	478	99.8	0.136	0.136	0.046	0.034	0.029	0.028	0.297	0.229	0.211	0.198	**0.508**	**0.427**
	19-64	2816	99.8	0.102	0.101	0.045	0.03	0.033	0.033	0.229	0.114	0.18	0.164	**0.409**	**0.278**
	>65	309	100	0.092	0.092	0.06	0.037	0.029	0.028	0.183	0.069	0.181	0.157	**0.364**	**0.226**
FINLAND	19-64	1432	90.9	0.125	0.114	0.127	0.073	0.048	0.039	-*	-*	0.3	0.226	**0.300**	**0.226**
	>65	432	93.3	0.124	0.116	0.139	0.073	0.034	0.025	-*	-*	0.297	0.214	**0.297**	**0.214**
FRANCE	11-18	844	86.7	0.074	0.064	0.047	0.035	0.038	0.037	0.229	-	0.159	0.136	**0.388**	**0.136**
	19-64	1599	70.3	0.047	0.034	0.059	0.041	0.052	0.05	0.143	-	0.158	0.125	**0.301**	**0.125**

	>65	167	63.26	0.042	0.026	0.054	0.033	0.057	0.056	-	-	0.153	0.115	**0.153**	**0.115**
GERMANY	11 18	602	59.6	0.084	0.049	0.065	0.017	0.04	0.03	0.247	-	0.189	0.096	**0.436**	**0.096**
	19-64	6011	57.7	0.062	0.035	0.081	0.029	0.049	0.039	0.4	-	0.192	0.103	**0.592**	**0.103**
	>65	989	49.3	0.054	0.026	0.08	0.033	0.047	0.04	0.178	-	0.181	0.099	**0.359**	**0.099**
HUNGARY	19-64	956	89.0	0.063	0.056	0.063	0.021	0.035	0.026	0.373	0.160	0.161	0.103	**0.534**	**0.263**
	>65	192	93.2	0.063	0.059	0.062	0.022	0.036	0.026	0.267	0.240	0.161	0.107	**0.428**	**0.347**
IRELAND	19-64	949	99.1	0.107	0.106	0.029	0.01	0.021	0.018	0.229	-	0.157	0.134	**0.386**	**0.134**
ITALY	11-18	210	85.0	0.071	0.06	0.052	0.011	0.065	0.063	-*	-*	0.188	0.134	**0.188**	**0.134**
	19-64	1742	75.3	0.054	0.041	0.056	0.014	0.066	0.064	-*	-*	0.176	0.119	**0.176**	**0.119**
	>65	224	77.2	0.057	0.044	0.052	0.01	0.058	0.056	-*	-*	0.167	0.11	**0.167**	**0.110**
LATVIA	11-18	231	49.2	0.055	0.027	0.082	0.033	0.036	0.022	0.2	-	0.173	0.082	**0.373**	**0.082**
	19-64	483	37.0	0.045	0.017	0.096	0.032	0.051	0.03	0.176	-	0.192	0.079	**0.368**	**0.079**
THE NETHERLANDS	19-64	481	64.1	0.111	0.071	0.136	0.067	0.049	0.04	0.4	0.160	0.296	0.178	**0.696**	**0.338**
SPAIN	11-18	612	94.0	0.132	0.124	0.091	0.066	0.029	0.017	0.264	0.132	0.252	0.207	**0.516**	**0.339**
	19-64	939	95.7	0.123	0.117	0.061	0.036	0.031	0.023	0.187	-	0.215	0.176	**0.402**	**0.176**
SWEDEN	11-18	952	93.5	0.133	0.124	0.08	0.041	0.019	0.012	0.286	0.190	0.232	0.177	**0.518**	**0.367**
	19-64	1041	86.0	0.109	0.094	0.094	0.058	0.033	0.031	0.107	-	0.236	0.183	**0.343**	**0.183**
UNITED KINGDOM	19-64	1673	97.0	0.086	0.084	0.033	0.014	0.025	0.022	0.111	0.012	0.144	0.12	**0.255**	**0.132**

* The applicant did not provide figures for the respective food categories for Italy and Finland. On request to EFSA, the applicant responded that the food categories available in the database did not match with the food products intended by the applicant. The figures for the intake estimate fro all food categories are therefore an underestimate for the two countries.