Conclusion of GRAS Status

of

Sodium Hyaluronate

Food Usage Conditions for General Recognition of Safety

on behalf of

Bloomage Biotechnology Corp., Ltd.
No. 678 Tianchen St., High-Tech Development Zone, Jinan, Shandong, 250101, China
and
No. 3333 Middle Century Avenue, High-tech Development Zone, Jinan, Shandong, 250101, China

10/23/2020

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PART 1. SIGNED STATEMENTS AND CERTIFICATION

At the request of Bloomage Biotechnology Corp., Ltd. ("Bloomage"), GRAS Associates, LLC ("GA") has determined that their sodium hyaluronate (NaHA) of molecular weight range 10-4000 kDa that is produced by microbial fermentation is generally recognized as safe, *i.e.*, GRAS, under the intended conditions of use, in accordance with Section 201(s) of the Federal Food Drug and Cosmetics Act[1]. This determination was made in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the conditions of the intended uses of this ingredient in foods.

Bloomage based its GRAS assessment on a large body of information that addressed the safety/toxicity of NaHA, history of use of NaHA and similar compounds and compositional details, specifications, and method of preparation of the subject ingredient.

Safety/toxicity studies performed with animals and human clinical trials that are cited in this document were noted to have value. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

In addition to the product specifications, chemical properties, manufacturing and safety-related information, Bloomage also provided consumption/exposure information, along with other related documentation. This was augmented with an independent search of the scientific and regulatory literature extending through May 25, 2020. A GRAS assessment based primarily on the composite safety information, *i.e.*, based on scientific procedures, was undertaken by Bloomage, followed by an Expert Panel review coordinated by GA. Those references that were deemed pertinent to the objective at hand are listed in Part 7B. The Expert Panel Report can be found in Appendix 1.

This signed statement and certification has been prepared in accordance with the requirements of 21 CFR 170.225[2].

(a) This certification will be signed at a future date by a responsible official of GRAS Associates, LLC acting as agent for Bloomage.

(b) This GRAS dossier did not rely on any confidential information;

(c) (1) This GRAS Assessment was conducted in accordance with Subpart E of 21 CFR Part 170[3];

(c) (2) Names and addresses of organizations;

Sponsoring Party:
Bloomage Biotechnology Corp., Ltd.
No 678 Tianchen St.
High-tech Development Zone
Jinan 250101
China

[1] https://www.law.cornell.edu/uscode/text/21/321
[2] https://www.law.cornell.edu/cfr/text/21/170.225
[3] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=170&showFR=1&subpartNode=21:3.0.1.1.1.5

Agent:
GRAS Associates LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

(c) (3) The name of the ingredient is sodium hyaluronate (NaHA). The Chemical Abstracts Registry Number name is β-D-Glucopyranuronic acid, 4-O-[2-(acetylamino)-2,3-dideoxy-β-D-ribo-hexopyranosyl], sodium salt (1:1). Synonyms for this substance are: Hyaluronate sodium, Hyalurone sodium and Hyaluronic acid sodium.

(c) (4) The ingredient will be used as an ingredient in beverages, including fruit drinks/ades and carbonated soft drinks, candy, milk and milk products, and ready-to-eat cereals. The NaHA that is the subject of this GRAS assessment is not proposed for uses in foods that are intended for infants, such as infant formulas or in any meat or poultry products that are regulated by the USDA.

(c) (5) The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with § 170.30(a) and (b).

(c) (6) It is our view that the ingredient is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

(c) (7) If FDA were to ask to see the data and information that are the basis for our conclusion of GRAS status, either during or after FDA evaluation of this notice, we agree to:

(i) make the data and information available to FDA

Contact:
William Rowe
GRAS Associates LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852; and

(ii) agree to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA's request, we will allow FDA to review and copy the data and information during customary business hours at our address specified where these data and information will be available; and

(B) Upon request by FDA, we will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for their evaluation or on paper.

(c) (8) We certify that, to the best of our knowledge, this GRAS Assessment is a complete, representative and balanced review that includes unfavorable information, as well as favorable information, known to us and is pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Bloomage does not intend to add NaHA to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

(c) (9) Signature

William Rowe
President
GRAS Associates LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

Date: 10/23/2020

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

A. Historical Information for Sodium Hyaluronate

Traditionally, hyaluronic acid (HA) for use in pharmaceuticals and foods was extracted from rooster combs or chicken sternal cartilage but is now mainly produced *via* streptococcal fermentation due to lower production costs and less environmental pollution (Liu et al., 2011). HA derived from a bacterial source exhibits superior reproducibility, excellent yields and a high degree of purity. The production of NaHA by *Streptococcus* was first demonstrated by Kendall (Kendall et al., 1937) and industrial scale microbial NaHA production was first achieved in the 1980s by Shiseido. *Streptococcus equi* subsp. *zooepidemicus* remains the current common strain for industrial production of NaHA (Chong and Nielsen, 2003; Krahulec and Krahulcova, 2006). Recent advances in the fermentation process with variants of *Streptococcus* and other organisms to produce NaHA and HA with customized properties have been reviewed (Liu et al., 2011).

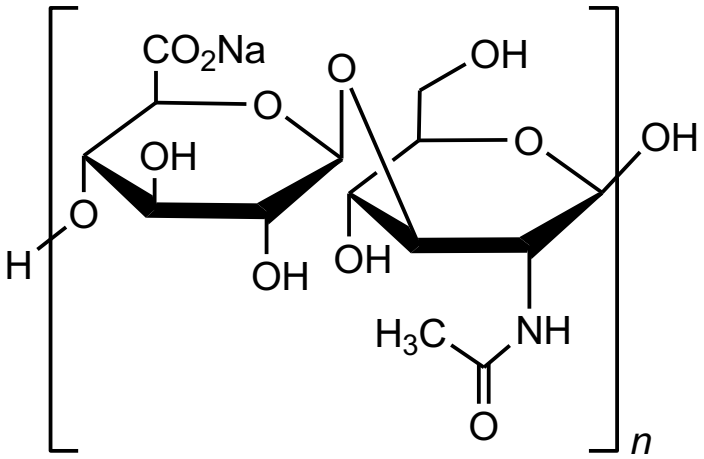
Molecular weight is an important specification for a commercial NaHA product as it determines the physiological response to NaHA while also helping to define the appropriate applications (Armstrong and Johns, 1997; Blank et al., 2008). NaHA with a molecular weight of greater than 10 kDa has good viscoelasticity, moisture retention and mucoadhesion; qualities that are desirable for ophthalmologic, orthopedic and cosmetic products. NaHA with a lower molecular weight in the range of 2-3.5 kDa has been shown to induce an angiogenic response in skin wounds and the expression of anti-inflammatory and inflammatory mediators involved in the healing process, as well as inhibition of tumor growth (Rahmanian et al., 1997; McKee et al., 1996; Zeng et al., 1998). One benefit of the microbial production of NaHA is that culture conditions can be altered to modify the molecular weight of the product. For example, culturing in a more aerobic environment can increase the molecular weight of NaHA due to increased energy availability compared to an anaerobic environment (Armstrong and Johns, 1997).

B. Chemistry of Sodium Hyaluronate

Sodium hyaluronate (NaHA) is the sodium salt of hyaluronic acid (HA)[4] (Food Chemicals Codex, 2018). HA is a natural, complex, non-sulfated carbohydrate of the glycosaminoglycan family. It is a long-chain polymer containing *d*-glucuronic acid and *N*-acetyl-*d*-glucosamine units linked by a β-1,3-glycosidic bond; these disaccharides are polymerized *via* β-1,4-glycosidic bonds. NaHA is a white or almost white, odorless, and very hygroscopic powder or fibrous aggregate. It is soluble in water; insoluble in acetone and in anhydrous ethanol.

The chemical structure of NaHA is shown in Figure 1.

Figure 1. Chemical Structure of Sodium Hyaluronate



1. Chemical Identity of Sodium Hyaluronate (NaHA)

Common or Usual Name: Sodium Hyaluronate (NaHA)
Chemical Name: Sodium salt of hyaluronic acid, a glycosaminoglycan consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide units.
Synonyms: Sodium salt of hyaluronic acid, Hyaluronate, Hyaluronan
CAS Number: 9067-32-7
Molecular Formula: $(C_{14}H_{20}NO_{11}Na)_n$
Molecular Mass: $(401.34)_n$
Molecular Weight Range: 10-4000 kDa

[4] "HA" is used to describe hyaluronic acid or sodium hyaluronate in many references. Sometimes it is not clear which form is being discussed. We have used in this evaluation "NaHA" when it is clear that the sodium form is being discussed. We have used "HA" for the acid form or when the form is unclear, and the referenced article used "HA."

2. Manufacturing Process for NaHA

a. Fermentation Strain

NaHA is manufactured by fermentation using the bacterial strain *Streptococcus equi* subsp. *zooepidemicus*, a gram-positive, non-motile, facultative anaerobe that is asporous (Bergey and Holt, 2000). The cells are spherical and occur as pairs or chains with colorless and transparent colonies. During the growth stage, HA is secreted, which is the main component of the bacterial capsule. *Streptococcus equi* subsp. *zooepidemicus* is Lancefield Group C of *Streptococci* (Bergey et al., 1974), which is specified as the fermentation bacteria for NaHA production by the European Pharmacopeia (European Pharmacopoeia, 2010), Japanese Pharmacopeia (Japanese Ministry of Health Labour and Welfare, 2016) and Korean Pharmacopeia (Korean Ministry of Food and Drug Safety, 2014).

The *Streptococcus equi* subsp. *zooepidemicus* production strain used by Bloomage is non-hemolytic. The parental strain was initially characterized (Farrow and Collins, 1984) and subsequently validated (Farrow and Collins, 1985) by Farrow and Collins. Bloomage originally purchased the strain from National Collection of Type Cultures. No antimicrobial resistance genes, including ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline or chloramphenicol, have been introduced into the strain. Additionally, no antimicrobials are used during strain storage or during the NaHA production process.

Physiological and biochemical characterization of Bloomage's *Streptococcus zooepidemicus* parental strain was conducted by the Institute of Microbiology of the Chinese Academy of Sciences (see Appendix 2) and 16S rRNA sequencing confirmed its identity as *Streptococcus equi* subsp. *zooepidemicus* (see Appendix 2).

Bloomage uses best industry practices in storing the mother culture and generating starter cultures of the production strain to assure genetic microbiologic purity of the organism and to avoid contamination by other organisms. Batch records assure traceability to the exact lot starter culture.

b. Fermentation, Purification and Refining

NaHA is manufactured under ISO9001:2015 and ISO22000:2005 by classical fermentation. A quaternary strain bank system has been established, including a primary strain bank, master strain bank, subculture strain bank and working strain. The manufacturing process of NaHA consists of three stages as shown in Figure 2: Fermentation, Purification and Refining.

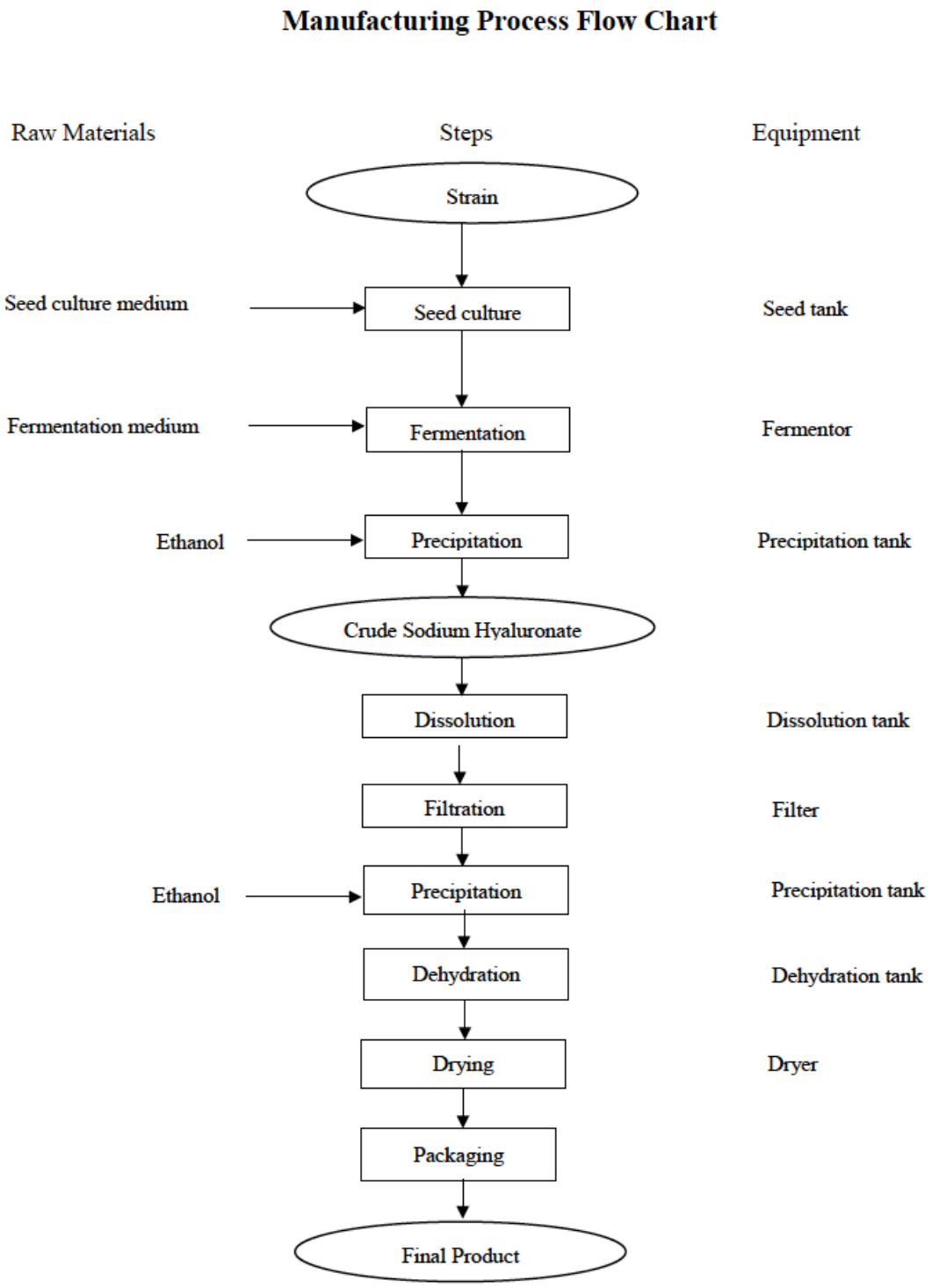
Fermentation: This step begins with the seed culture. The starter culture is used to inoculate the seed tank containing a sterilized broth medium and is grown out to become the seed broth. The seed broth is then transferred to a fermenter containing the sterilized fermentation medium and maintained at culturing temperature until fermentation is complete.

Purification: The fermentation broth is mixed with ethanol to obtain precipitated crude NaHA. The crude product is then dissolved in drinking water and filtered to remove impurities and inactivated microbial fragments to yield a clear filtrate.

Refining: The filtrate from the purification step is subjected to precipitation and dehydration with ethanol, respectively. The precipitate is dried under vacuum to yield the final NaHA product.

A manufacturing process flow chart for production of Bloomage's NaHA is provided in Figure 2.

Figure 2. Manufacturing Process Flow Chart for Bloomage's NaHA



C. Product Specifications

Bloomage manufactures NaHA under ISO9001:2015 and ISO22000:2005 requirements, using culture media containing glucose, yeast extract, peptone, potable water and sequestrants and processing aids permitted for use in food and of a purity suitable for the intended use. Analytical testing for 380 pesticide residues was performed for each of the three major raw materials, and all were determined to be less than the limits of detection (pesticide testing data is available in Bloomage's files).

1. Specifications for NaHA Compared to FCC/21 CFR Specifications

Bloomage has established specifications and confirmed compliance with these specifications for its food-grade NaHA. Bloomage's specification/product data sheet and testing methods for NaHA are found in Appendix 3. There is an established standardized specification for NaHA (from microbial fermentation) per FCC/21 CFR (Food Chemicals Codex, 2018); therefore, Bloomage's NaHA was compared with FCC/21 CFR specifications as shown in Table 1. Bloomage produces food grade NaHA of various molecular weights based on customer needs. Bloomage's MW range is slightly broader (10-4000 kDa) than the FCC MW range (80.2-4010 kDa) (Food Chemicals Codex, 2018). In 2014, Bioiberica S.A. received a "no questions" letter from FDA from its GRN No. 491 submission for the use of Rooster Combs Extract (RCE) as an ingredient in food (Bioiberica S.A., 2014). RCE contains 60-80% w/w NaHA as well as other components that include, chondroitin sulfate, dermatan sulfate, proteins, fiber and free amino acids. Bioiberica S.A.'s specifications for RCE are also included in Table 1.

Table 1. Bloomage Biotechnology Corp.'s Specifications for NaHA Compared to the FCC 11 Method

Physical and Chemical Parameters	FCC Sodium Hyaluronate (from Microbial Fermentation)[1]		Bloomage Biotechnology Corp.'s Sodium Hyaluronate[2]		Bioiberica S.A.'s Rooster Comb Extract (GRN No. 491)[3]
	Method	Specification	Method[4]	Specification	Specification
Character	Visual	White or almost white hygroscopic powder	Visual	White or almost white powder granules	White or almost white hygroscopic powder
Identification – IR absorption	IR spectrophotometric identification tests	Consistent with reference spectrum of NaHA	IR spectrophotometric identification tests	Consistent with reference spectrum of NaHA	NR
Identification – Reaction of sodium	Sodium identification test	Passes test	Reaction of Sodium	Positive	NR
Sodium hyaluronate	Sodium hyaluronate content assay (USP *d*-glucuronic acid RS)	NLT 95%	Sodium hyaluronate content assay	≥ 93.0%	60-80%
pH	pH determination	6.0~8.0	pH determination	6.0~8.0	5.0~8.5

Physical and Chemical Parameters	FCC Sodium Hyaluronate (from Microbial Fermentation)[1]		Bloomage Biotechnology Corp.'s Sodium Hyaluronate[2]		Bioiberica S.A.'s Rooster Comb Extract (GRN No. 491)[3]
	Method	Specification	Method[4]	Specification	Specification
Loss on drying	Water determination	NMT 20%	Water determination	≤ 10.0%	≤ 10%
Molecular weight	NS	$8.02x10^4$ to $4.01x10^6$ Da	Intrinsic viscosity testing method	10-4000 kDa	NR
Intrinsic viscosity	Intrinsic viscosity testing method	90-120 % labeled value		NR	NR
Ash	NS	NS	Ignition at 700-800 °C for 1.5 h	≤ 13.0%	NR
Inorganic Impurities					
Lead	Flame atomic absorption spectrophotometric method	NMT 1 mg/kg	Flame atomic absorption spectrophotometric method	≤ 1 ppm	≤ 0.5 ppm
Arsenic	Arsenic limit test	NMT 2 mg/kg	Chinese Ph. 4.0822.1	≤ 2 ppm	NR
Chloride	Chloride limit test	NMT 0.5%	NR	NR	NR
Microbiological Limits					
Bacteria counts (Total viable aerobic count)	NS	NS	Chinese Ph. 4.1105	≤ 500 cfu/g	≤ 100 cfu/g
Molds & Yeast (Total viable aerobic count)	NS	NS	Chinese Ph. 4.1105	≤ 100 cfu/g	
E. coli	NS	NS	Chinese Ph. 4.1106	Negative	Absent/g
Staphylococcus aureus	NS	NS	Chinese Ph. 4.1106	Negative	Absent/g
Salmonella sp.	NS	NS	Chinese Ph. 4.1106	Negative	Absent/g

[1] Food Chemicals Codex (2018)
[2] Based on information provided by Company
[3] Bioiberica S.A. (2014)
[4] Bloomage's methods are included in Appendix 3
Abbreviations: NS – Not specified; NR – Not reported; NLT – Not less than; NMT – Not more than; IR – Infrared; cfu – Colony Forming Unit; PPM – parts per million

2. Nutritional Profile for Bloomage's NaHA

Table 2 below shows a typical nutritional profile for NaHA. The Certificate of Analysis is provided in Appendix 4.

Table 2. Typical Nutritional Profile for NaHA[1]

COMPONENT	TYPICAL ANALYSIS OF SODIUM HYALURONATE (BATCH 1403252)
Iron	1.37 mg/kg
Calcium	127 mg/100 g
Sodium	5800 mg/100 g
Total Fat	<0.1 g/100 g
Cholesterol	ND
Fatty Acid (FA) Profile	
Mono-unsaturated FA	ND
Poly-unsaturated FA	ND
Saturated FA (total)	ND
Trans FA (total)	ND

[1] Eurofins Technology Services (Suzhou) Co., Ltd.
Abbreviations: FA – Fatty Acid, ND – not detected

3. Batch Results for Bloomage's NaHA and Supporting Methods

The composition of six non-consecutive batches of NaHA, as well as product specifications, are provided in Table 3. Bloomage has provided Certificates of Analysis (COA) for 6 non-consecutive batches of NaHA (see Appendix 5; note that that the company changed its name from Bloomage Freda Biopharm. Co., Ltd. to Bloomage Biotechnology Corp., Ltd. on March 6, 2019) as representative products to demonstrate that the food grade specifications outlined in Table 1 are met.

The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

Table 3. Specifications and COAs of Bloomage Biotechnology Corp.'s NaHA

PHYSICAL AND CHEMICAL PARAMETERS	BLOOMAGE SPECIFICATION	SODIUM HYALURONATE BATCH RESULTS					
		1603073	**1603218**	**1706224**	**1803313**	**1809191**	**1903208**
Character	White or almost white powder or granules	Complied	Complied	Complied	Complied	Complied	Complied
Identification – IR absorption	Consistent with reference spectrum of NaHA	Complied	Complied	Complied	Complied	Complied	Complied
Identification – Reaction of sodium	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Sodium Hyaluronate	≥ 93.0%	95.5%	95.4%	96.4%	96.0%	95.5%	96.7%
pH	6.0~8.0	6.2	6.3	6.5	6.4	6.4	6.2
Loss on Drying	≤ 10.0%	7.7%	7.0%	6.8%	7.5%	5.2%	6.6%

Physical and Chemical Parameters	Bloomage Specification	Sodium Hyaluronate Batch Results					
		1603073	1603218	1706224	1803313	1809191	1903208
Molecular weight (kDa)	10-4000	1370	510	1060	1320	530	380
Ash	≤ 13.0%	10.3%	10.1%	10.1%	11.9%	11.2%	9.4%
Inorganic Impurities							
Lead	≤ 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm	< 1 ppm
Arsenic	≤ 2 ppm	< 2 ppm	< 2 ppm	< 2 ppm	< 2 ppm	< 2 ppm	< 2 ppm
Microbiological Limits							
Bacteria Counts	≤ 500 cfu/g	Complied	Complied	Complied	Complied	Complied	Complied
Yeast and Molds	≤ 100 cfu/g	Complied	Complied	Complied	Complied	Complied	Complied
E. coli	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Staphylococcus aureus	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella sp.	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Abbreviations: cfu – Colony Forming Unit; ppm – parts per million

D. Physical or Technical Effect

Bloomage has proposed the use of NaHA as an ingredient in food and beverages, including fruit drinks/ades and carbonated soft drinks, milk and milk products, and ready-to-eat cereals. The addition of NaHA to these products would be intended for those individuals wishing to increase their daily intake of HA. Bloomage estimates the mean and 90th percentile estimated dietary intakes at the proposed maximum intended use levels of NaHA to be 125 and 250 mg NaHA/day, respectively (see Table 6).

1. Stability Data for Bloomage's NaHA

The stability of NaHA was investigated under long-term storage conditions (*i.e.*, 25 ± 2°C; 60 ± 5% relative humidity). Three batches of HA were tested, and specification parameters considered susceptible to change over time were measured after 0, 6, 12, 18, 24 or 36 months under long-term storage conditions. Results of the long-term stability test indicate that all measured parameters remained within Bloomage's specification limits for NaHA over the duration of the storage period. Long-term stability data by batch at 0, 24 and 36 months is presented in Table 4. An accelerated stability study (*i.e.*, 40 ± 2°C; 75 ± 5% relative humidity) was conducted at 0, 1, 2, 3 and 6 months and is presented in Table 5. Test results from the long-term and accelerated stability studies demonstrate that Bloomage's NaHA shows little variability under these conditions.

Based on the data obtained from stability studies and on the production experience for NaHA, the shelf-life of Bloomage's NaHA was determined to be three years when maintained under the prescribed conditions.

Table 4. NaHA Ambient Condition Long-term Storage Stability Data (25 ± 2°C; 60 ± 5% relative humidity)

Physical & Chemical Parameters	Acceptance Criteria	1207202			1411183			1507025		
		0 Mos.	24 Mos.	36 Mos.	0 Mos.	24 Mos.	36 Mos.	0 Mos.	24 Mos.	36 Mos.
Character	White or almost white powder or granules	Complied	Complied	Complied	Complied	Complied	Complied	Complied	Complied	Complied
pH	6.0~8.0	6.6	6.9	6.4	6.3	6.4	6.4	6.5	6.7	6.5
MW (kDa)	10-4000	500	480	470	390	400	420	350	300	330
LOD (%)	≤ 10.0	6.1	5.8	6.4	5.8	8.2	8.0	6.9	8.0	9.5
NaHA (%)	≥ 93.0	95.9	96.5	96.8	96.7	95.9	96.3	96.5	95.4	96.6
Microbiological Limits										
Bacteria Counts (cfu/g)	≤ 500	<10	<10	< 10	< 10	<10	< 10	< 10	<10	< 10
Mold & Yeasts (cfu/g)	≤ 100	<10	<10	< 10	< 10	<10	< 10	< 10	<10	< 10
E. coli	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
S. aureus	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Salmonella sp.	Negative	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Abbreviations: cfu – Colony Forming Unit; LOD – Loss on Drying; Mos. – months; NaHA- Sodium Hyaluronate; ppm - parts per million; NA – Not Available; Neg – Negative; ppm – parts per million

Table 5. NaHA Accelerated Condition Stability Data (40 ± 2°C; 75 ± 5% Relative Humidity)

Physical & Chemical Parameters	Acceptance Criteria	1104302		1105123		1105131	
		0 Mos.	6 Mos.	0 Mos.	6 Mos.	0 Mos.	6 Mos.
Character	White or almost white powder or granules	Complied	Complied	Complied	Complied	Complied	Complied
pH	6.0~8.0	6.49	6.61	6.36	6.20	6.44	6.27
MW (kDa)	10-4000	330	270	350	300	340	310
LOD (%)	≤ 10.0	6.24	7.82	5.91	7.89	6.26	7.90
NaHA (%)	≥ 93.0	96.8	96.3	96.6	96.6	95.1	96.6
Ash (%)	≤ 13.0	10.1	10.2	10.5	10.4	10.2	10.3
Microbiological Limits							
Bacteria Counts (cfu/g)	≤ 500	Complied	Complied	Complied	Complied	Complied	Complied
Mold & Yeasts (cfu/g)	≤ 100	Complied	Complied	Complied	Complied	Complied	Complied

Abbreviations: cfu – Colony Forming Unit; LOD – Loss on Drying; Mos. – months; NaHA – Sodium Hyaluronate; ppm - parts per million

PART 3. DIETARY EXPOSURE

A. Current Dietary Exposure to NaHA

HA is endogenous to all living organisms and is widely distributed in tissues and intracellular fluids (Lebel, 1991; Necas et al., 2008). HA is found in high concentrations in several soft connective tissues, including skin, umbilical cord, synovial fluid and vitreous humor. Significant amounts are also found in lung, kidney, brain and muscle tissues (Fraser et al., 1997). HA is a common natural component of food. It is present in animal products where bone is present and would be extracted when present in soups or stews. Rooster combs, which are high in HA, are a popular gourmet item in European countries like France and Spain and are used as a typical ingredient in many dishes like homemade chicken soup, stews and traditional dishes (Bioiberica, 2010).

Although numerical estimates of background dietary intake of NaHA or HA were not identified, data on blood and tissue concentrations in several animals are available. Serum or plasma concentrations in sheep and pigs range from 100 to 260 ng/ml (Lebel, 1991) and the concentration of HA in skeletal muscle of rabbits is reported to be 26 to 28 μg/g (Necas et al., 2008). The concentration of HA in the skin of rabbits is much higher at 840 μg/g. Humans are exposed to endogenous background levels due to intracellular HA synthesis in the Golgi network by the integral membrane proteins, hyaluronan synthases. The total amount of HA in the human body is estimated at 14-16 g with half of that located in the skin (Becker et al., 2009). Normal concentrations in the plasma of healthy human volunteers are much lower with a range of 10-100 ng/ml and a mean value of 30-40 ng/ml (Lebel, 1991). The normal daily turnover of HA in humans is 34 mg/day.

B. Intended Food Uses of Bloomage's NaHA (Estimated Daily Intake)

Bloomage's NaHA will be added as an ingredient in conventional foods. The proposed products and use levels per serving are:

- 60 mg NaHA in non-alcoholic beverages, including fruit drinks and ades;
- 50 mg NaHA in non-alcoholic beverages, including carbonated soft drinks;
- 60 mg NaHA in candies;
- 40 mg in milk and milk products, including milk, milk drinks and yogurt; and
- 40 mg in grain products, including ready-to-eat cereals weighing 43 g or more per cup (biscuit types).

The daily intake of NaHA is estimated using proposed intended use levels and mean consumption estimates of each food category reported by the National Health and Nutrition Examination Survey (NHANES) 1994-96 survey. Based on these studies reporting daily consumption, the mean and high (90th percentile) consumption of Bloomage's NaHA from the above-mentioned proposed food uses were calculated. In order to estimate the 90th percentile consumption of Bloomage's NaHA, the corresponding mean total intake value was multiplied by two on the grounds that the 90th percentile consumption is unlikely to exceed the mean by more than a factor of two (FDA, 2006). This FDA methodology is recognized as a method that overestimates consumption. Using NHANES estimated mean intakes of the food categories for which Bloomage's NaHA are proposed to be added, the possible mean and maximum daily intake of Bloomage's NaHA based on all individuals can be found in Table 6. Bloomage estimates the mean and high (90^{th} percentile) estimated dietary intakes at the proposed maximum intended use levels of NaHA to be 125 and 250 mg NaHA per day, respectively.

In 2014, Bioiberica S.A.'s GRN No. 491 for the use of RCE as an ingredient in food received a "no questions" response from FDA (Bioiberica S.A., 2014). Bioiberica's RCE (IB0004) consists of approximately 60-80% NaHA, 20% glycosaminoglycans and 20% partially hydrolyzed proteins (see Table 7). A 13-wk subchronic toxicity study of Bioiberica's RCE in rats calculated a no adverse effect level (NOAEL) of 600 mg RCE/kg/day (Canut et al., 2012). If this NOAEL is multiplied by 60% NaHA, a NOAEL of 360 mg NaHA/kg bw/day in rats is obtained. Additionally, the Japanese Health Food and Nutrition Food Association issued a food industry standard for HA and affirmed the safety of HA in foods, setting a maximum daily intake of 250 mg HA per person or 3.5 mg/kg bw/day for a 70 kg human (Japanese Health Food and Nutrition Food Association, 2011). Bloomage proposes that a 90th percentile use level of 250 mg NaHA per day is GRAS because it would provide a 100x safety factor between the dose in humans and the NOAEL of 360 mg NaHA/kg bw/day in rats.

Foods with added NaHA would replace those containing RCE and, therefore, would not be an additive amount. NaHA is a common dietary supplement ingredient and there is a similar expectation that individuals consuming dietary supplements containing NaHA would not also be high consumers of conventional foods containing added NaHA. The NaHA that is the subject of this GRAS assessment is not proposed for uses in foods that are intended for infants, such as infant formulas or in any meat or poultry products that are regulated by the USDA.

Table 6. Summary of All Individual Proposed Food Uses & Use Levels for Bloomage's NaHA in the US Based on the National Health and Nutrition Examination Survey (NHANES) [1994-96]

Food Category[1]	Sub-category	Maximum NaHA per Serving (mg)	Mean [g/day] of Food Consumed (All Individuals)[2]	Serving Size (g)	RACC[3] # Servings Per Day	Mean Intake NaHA mg/day consumed (All Individuals)	
						Mean	Mean x 2
Beverages, nonalcoholic	Fruit drinks and ades	60.0	95	360	0.2639	15.834	31.668
	Carbonated soft drinks	50.0	332	360	0.9222	46.11	92.22
Candy	All other candies	60.0	7	30	0.2333	14.000	28.000
Milk & milk products	Milk, milk drinks, yogurt	40.0	227	240	0.9458	37.832	75.664
Grain products	Ready-to-eat cereals; weighing 43 g or more/cup; biscuit types	40.0	16	60	0.2667	10.668	21.3336
						124.444	**248.888**

[1] 21CFR 170.3(n)
[2] Data from Results from the USDA's 1994-96 Continuing Survey of Food Intakes by Individuals and 1994-96 Diet and Health Knowledge Survey Table Set 10 (https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/Csfii3yr.pdf)
[3] Reference Amount Customarily Consumed (RACC) in https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/labelingnutrition/ucm513820.pdf)

C. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed in or on Food

Not applicable.

D. Dietary Exposure to Contaminants, Byproducts and Other Bioactives

Potential contaminants of Bloomage's NaHA include microbes and heavy metals. The specifications set for Bloomage's NaHA place limits on the maximum permissible levels of these impurities to assure an acceptable final product. The batch data for 6 different lots document quality control of the final product such that it meets these specifications (Table 3).

PART 4. SELF-LIMITING LEVELS OF USE

The use of Bloomage Biotechnology Corp.'s NaHA will be self-limiting in beverage products due to its viscosity.

PART 5. EXPERIENCE BASED ON COMMON FOOD USE IN FOOD BEFORE 1958

HA and NaHA are present naturally in foods and were present in foods prior to 1958. However, the statutory basis for the conclusion of GRAS status of NaHA in this document is not based on common use in food before 1958. The GRAS conclusion is based on scientific procedures.

PART 6. NARRATIVE

A. Regulatory History of NaHA

1. United States

Sodium hyaluronate (NaHA) is the sodium salt of hyaluronic acid (HA). The first hyaluronan biomedical product, Healon® (sodium hyaluronate 10 mg/ml), was developed by Pharmacia and marketed in 1980 (Higashide and Sugiyama, 2008). It was approved by US Food and Drug Administration (FDA) as a topical product for use as an adjunct to ophthalmic surgery during cataract extraction, corneal transplant, intraocular lens implantation, glaucoma filtration and retina attachment surgery by assisting to maintain spaces, move tissue and protect surfaces (Higashide and Sugiyama, 2008). Other biomedical companies now also produce brands of hyaluronan for ophthalmic surgery. Hyaluronic acid and derivatives have also become popular agents used for soft tissue augmentation in nonsurgical facial rejuvenation (Rohrich et al., 2007). As of 2017, FDA has approved 13 hyaluronate preparations for use as dermal fillers. Intra-articular administration of exogenous HA is used to treat knee pain in patients with osteoarthritis who have failed to respond adequately to conservative non-pharmacologic therapy and to certain analgesics (Moreland, 2003). It is generally used as a last resort before surgery, provides symptomatic relief and may delay joint replacement. Currently, NaHA is an ingredient in 522 dietary supplements on the market in the US (Office of Dietary Supplements, 2019).

A monograph characterizing sodium hyaluronate produced from microbial fermentation (specifies *Streptococcus*) was published in the Food Chemical Codex (11th ed.) (Food Chemicals Codex, 2018). A monograph for HA (including NaHA) is also included in the Natural Medicines Comprehensive Database (Therapeutic Research Faculty, 2018).

A search of FDA's GRAS Notice Inventory website[5] found one notification related to sodium hyaluronate that is extracted from rooster combs. This GRAS Notice (GRN) is summarized in Table 7. Bioiberica S.A.'s GRN No. 491 for the use of Rooster Combs Extract (RCE) as an ingredient in food received a "no questions" response from FDA (Bioiberica S.A., 2014). No GRNs for NaHA or HA from any other sources were identified.

Table 7. Summary of Rooster Combs Extract (RCE) GRN No. 491

SUBSTANCE	GRN # / CLOSURE DATE	INTENDED USE	USE RATE	COMPANY/ REFERENCE
Rooster Combs Extract 60-80% (w/w) NaHA ≤ 5% (w/w) chondroitin sulfate ≤ 25% (w/w) dermatan sulfate ≤ 25% (w/w) protein < 1% fiber < 2 % free amino acids	491/ June 6, 2014	As an ingredient in food: Baking goods & mixes Beverages & beverage bases Breakfast cereals Cheeses Dairy product analogs Grain products & pastas Milk & milk products Processed fruits & juices Medical foods	80 mg/serving 2 servings/day	(Bioiberica S.A., 2014)

Abbreviations: w/w – weight-weight

2. Europe

In February 2011, Bioiberica SA initiated a novel food ingredient application for RCE to be used in dairy products. Bioiberica SA made a request to the authorities of the UK and after getting an initial positive assessment report, the Commission consulted the European Food Safety Authority (EFSA) asking it to carry out an assessment for RCE as a food ingredient in accordance with Regulation (EC) No 258/97. In May 2013, EFSA adopted a Scientific Opinion that concluded RCE is safe under the proposed use in dairy products at a maximum dose of 80 mg/day (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013). The Scientific EFSA Opinion gave sufficient grounds to establish that RCE in the proposed uses and use levels complies with the criteria laid down in Article 3(1) of Regulation (EC) No 258/97. In December 2013, the Commission Implementing Decision of 29 November 2013 was adopted (European Commission, 2013) and this authorized the placing on the market of RCE as a novel food ingredient under regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2013) 8319) (2013/705/EU)).

3. Japan

In 1996, the Japanese Ministry of Health, Labour and Welfare evaluated the safety of HA extracted from rooster combs or obtained by fermentation with *Streptococcus zooepidemicus*. HA was deemed safe for food use and was subsequently listed in the Japanese Food Additives Catalog (Japanese Ministry of Health Labour and Welfare, 1996). In 2011, the Japanese Health Food and Nutrition Food Association issued a food industry standard for HA, specifying the production and analytical methods for HA and affirming the safety of HA in foods, setting a maximum daily intake of 250 mg per person

[5] https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices (accessed 5/29/19)

(Japanese Health Food and Nutrition Food Association, 2011). Currently, NaHA is listed in The Japanese Pharmacopoeia (Japanese Ministry of Health Labour and Welfare, 2016) and HA-containing foods are classified as either health foods or conventional foods. Heath foods include capsules and powders such as HA collagen powder, which can be added to beverages. The conventional foods in Japan containing HA include drinks, yogurt, chewing gum, jams, tea powder, candy and honey.

4. South Korea

In Korea, HA is approved as a food additive by its Ministry of Food and Drug Safety. In 2014, standards and specifications for the production of HA were enacted (Korean Ministry of Food and Drug Safety, 2014). Products that contain HA sold on the Korean market include HA-containing capsules and beverages. CJ Corporation (Los Angeles, CA, USA), the largest food company in Korea, markets health foods and beverages containing HA. In addition, Lotte Group (Seoul, South Korea) markets a beverage containing HA, and Namyang Dairy Products Co., Ltd (Seoul, South Korea) offers a line of tea drinks containing HA.

5. People's Republic of China

In 2008, the Ministry of Heath of the People's Republic of China approved HA for use in foods and as a new resource for use in health foods at a daily dose not exceeding 200 mg per person (Ministry of Health of the People's Republic of China, 2008). In 2012, a combination of HA and marine fish collagen peptide powder (Beijing Tong Ren Tang Heath Pharmaceutical Co., Ltd) was approved by the State Food and Drug Administration. The HA used in this product is supplied by Bloomage.

6. Canada

HA (purified or from bacterial fermentation) is classified as a Natural Health Product under Schedule 1, item 2 (an isolate) of the Natural Health Products regulations[6].

B. Discussion of the Safety of NaHA and HA

Numerous published studies have investigated the metabolism and safety of NaHA and HA from various sources by oral and other routes of administration. Two formulations in particular have been studied in detail, including a 1800-2100 kDa NaHA product (SL-1010) that is produced by fermentation using bacteria (species and strain not specified but presumably *Streptococcus zooepidemicus*) and a rooster comb extract (RCE) product. In 2010, Bioiberica S.A. (Barcelona, Spain) submitted an application for a novel food ingredient in the European Union for the authorization for a RCE product containing NaHA for use in dairy products (Bioiberica, 2010; EFSA Panel on Dietetic Products Nutrition and Allergies, 2013). In 2014, Bioiberica S.A.'s GRN No. 491 for the use of RCE as an ingredient in food received a "no questions" response from FDA (Bioiberica S.A., 2014). Bioiberica's RCE (IB0004) consists of approximately 60-80% sodium hyaluronate, 20% glycosaminoglycans and 20% partially hydrolyzed proteins (see Table 7). This NaHA has a molecular weight of approximately 800 kDa. In addition, several unpublished *in vitro*, animal and human studies were conducted by Bloomage and provide corroborative evidence that Bloomage's

[6] https://laws-lois.justice.gc.ca/eng/regulations/SOR-2003-196/ (accessed 6/10/19)

manufacturing process and extended molecular weight range do not change the safety profile of the material.

The summaries below concentrate on studies done *via* oral administration as that is most germane to the assessment of safety for food use. However, some studies conducted by other routes of administration will be discussed when the results provide additional insight on food safety considerations. Studies of both the acid and salt form are relevant. Numerous studies are available in Japanese only; English-language abstracts or summaries of these studies presented in English-language reviews were used (Becker et al., 2009; Necas et al., 2008).

1. Absorption, Distribution and Metabolism of NaHA and HA

Hyaluronic acid is an endogenous constituent of the connective tissues of vertebrates. HA is continually synthesized in peripheral tissues by a class of integral membrane proteins, the hyaluronan synthases (HAS), of which vertebrates have 3 types HAS1, HAS2, and HAS 3 (Prehm, 1983a; Prehm, 1984). The HAS lengthen HA by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded via the ABC-transporter through the cell membrane into the extracellular space (Prehm, 1983b; Prehm, 2006). Fasciacytes are the fibroblast-like cells that synthesize HA (Stecco et al., 2018).

The initial step of the catabolic process is the entrance of HA into the blood circulation through the lymph. It is then rapidly taken up by sinusoidal liver endothelial cells via receptor-mediated endocytosis and is degraded by a family of enzymes called the hyaluronidases (Laurent and Fraser, 1992). The half-life of HA in human blood is 2.5-5.5 minutes (Fraser et al., 1984). Metabolism is saturable and the maximum metabolic capacity for humans is estimated to be 350 mg/day with the normal daily turnover of HA approximately 34 mg/day (Lebel, 1991). HA is initially broken down to the monosaccharides, glucuronic acid and *N*-acetylglucosamine, that are further metabolized to end products that may include lactate, H_2O and CO_2. Approximately 1 to 20% of the daily turnover of HA in humans is filtered by the kidneys and end products in the form of H_2O or monosaccharide metabolites may be present in the urine (Fraser et al., 1984). Normal human serum levels are 10-100 ng/ml with elevated levels of HA observed in liver cirrhosis, rheumatoid arthritis and scleroderma resulting both from impaired catabolism in the liver and an increased synthesis in the peripheral tissues (Laurent et al., 1986). Intravenous administration of HA into pregnant and lactating rats showed little transfer of the material to the fetus or milk (Iwata et al., 1991).

Due to the high average molecular weight of endogenous human HA (~1000 kDa) (Bucci, 2004), and its rapid clearance from the bloodstream by the liver (Laurent and Fraser, 1992), it was initially assumed that oral HA would exhibit poor tissue bioavailability (Balogh et al., 2008). However, an investigation of the uptake of ^{99m}Tc-HA (>1000 kDa, food grade NaHA (Nutrihyl® (Contipro a.s.)) in the rat and dog after oral ingestion provided evidence of uptake and distribution into connective tissues, namely the skin, bone, muscle and joints, but not other organs in both animal models (Balogh et al., 2008). Average total excretion of ^{99m}Tc-HA in rats over a 72 h period were 84.6 $\pm$ 7.8% of the ingested dose in feces and 2.0 $\pm$ 0.63% of the ingested dose in urine, for a total urine plus feces excretion of 86.7 $\pm$ 8.0% of the ingested dose (Balogh et al., 2008). A small percentage, approximately 0.1-10% of the administered dose, accumulated in blood; bone; vertebra; shoulder, sternocostal, and knee joints; muscle; salivary glands; and skin. In a separate study, oral administration of ^{99m}Tc-HA of three different MW (100 kDa, 0.5 MDa or 1 MDa) to rats resulted in only trace to negligible radioactivity levels in the bloodstream, organs and tissues (Laznicek et al., 2012).

The plasma clearance, tissue distribution and metabolism of HA was investigated in rabbits using a high average molecular weight [^{3}H]acetyl-labeled HA (Fraser et al., 1981). Following intravenous injection in the rabbit, the label disappeared from the plasma with a half-life of 2.5-4.5 min corresponding to a normal HA clearance of approximately 10 mg/kg bw/day. Approximately 88% of the label was absorbed by the liver, where it was found almost entirely in non-parenchymal cells. Degradation of HA was rapid and complete, as volatile material, presumably ^3H_2O, appeared in the plasma within 20 min. Undegraded [3H]HA, small labeled residues and ^3H_2O were detected in the liver, but there was little evidence of oligosaccharide intermediates. In plasma or urine, no metabolite except ^3H_2O was noted. After 24 hours of administration, two-thirds of the radioactivity was found in the body water, and small amounts were found in liver lipids. The upper molecular weight limit for renal excretion was about 25 kDa. Renal excretion played a negligible part in clearance. The investigators concluded that HA is removed from the plasma and degraded quickly by an efficient extrarenal system with a high reserve capacity, mainly in the liver.

In a study on the absorption of HA (MW unknown; Shangdong Freda Biochem Company (now Bloomage)), forty female Wistar rats received either a single oral dose of 60 mg HA/kg bw and either D-glucuronic acid or N-acetylglucosamine (doses not reported) or HA and an equimolar ratio of both the monosaccharides (Jiang et al., 2005). The concentration of HA in serum was determined by radioimmunoassay. No changes in serum HA levels were noted in the saline control group and D-glucuronic acid group. The serum HA levels in the HA group peaked at 2 h after oral administration while the serum HA levels from the other monosaccharide groups peaked about 7 h after oral administration.

The gastric absorption of a combination HA and phospholipids (PL) was studied in rats to determine the improvement effect of PL on the oral absorption of HA (Huang et al., 2007). Absorption was determined using radioimmunoassay to determine the concentration of HA in the blood serum. HA was obtained from Shandong Freda Biochem Company (now Bloomage). The molecular weight of the HA was not specified. The combination of HA-PL (Haplex) was prepared by film dispersion/sonication and 60 mg/kg bw was administered intragastrically to healthy female Wistar rats (N=8). Additional groups of rats (N=8) received saline (control), 60 mg/kg bw HA or a mixture of HA and PL prepared by mortar grinding. A peak in HA concentration was observed 4-12 h after intragastric administration of HA, Haplex and the HA-PL mixture. The serum concentration of HA increased after Haplex administration ($\Delta AUC_{0\text{-}12\ h}$ 777.9 $\pm$ 318.3 ng·h/ml) compared to the mixture of HA and PL ($\Delta AUC_{0\text{-}12\ h}$ 451.6 $\pm$ 401.3 ng·h/ml), HA alone ($\Delta AUC_{0\text{-}12\ h}$ 381.8 $\pm$ 340.8 ng·h/ml) or the saline control groups.

2. Biological Activity of NaHA and HA

The biological functions of HA include maintenance of the elastoviscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, supramolecular assembly of proteoglycans in the extracellular matrix and numerous receptor-mediated roles in cell detachment, mitosis, migration, tumor development and metastasis, and inflammation (Necas et al., 2008). The unique viscoelastic nature of HA along with its biocompatibility and non-immunogenicity has led to its use in a number of clinical applications, including the supplementation of joint fluid in arthritis, as a surgical aid in eye surgery and to facilitate the healing and regeneration of surgical wounds.

Molecular weight is an important specification for a commercial HA product because it determines the physiological response to HA and defines the appropriate applications (Armstrong and Johns, 1997; Blank et al., 2008). HA with a molecular weight greater than 10 kDa exhibits good viscoelasticity, moisture retention and mucoadhesion qualities that are desirable for ophthalmologic, orthopedic and cosmetic products. HA with a low molecular weight of 2-3.5 kDa or HA oligosaccharides (10-20 sugars in length) have been shown to induce angiogenesis (Rahmanian et al., 1997) and expression of inflammatory mediators (McKee et al., 1996), as well as inhibit tumor growth (Zeng et al., 1998).

3. *In vitro* Safety Studies with NaHA and HA

Bacterial reverse mutation assays, mouse micronucleus testing, sperm malformation testing and chromosomal aberration assays have been performed to evaluate the mutagenic potential of NaHA and RCE.

Sodium hyaluronate

A bacterial reverse mutagenicity test, a mouse micronucleus test and a sperm malformation test were performed as part of a comprehensive safety study of NaHA (Zuo et al., 2008). NaHA did not exhibit any significant mutagenic activity in *Salmonella* strains TA97a, TA98, TA100 or TA102 ±S9 activation at concentrations of 8 μg/plate, 40 μg/plate, 200 μg/plate, 1000 μg/plate or 5 mg/plate. For the micronucleus test, five groups of 10 mice were administered 0, 1,250, 2,500 or 5,000 mg NaHA/kg bw *via* oral gavage twice at an interval of 24 h. Test groups had no significant differences in micronucleated PCEs compared to the negative control. For the sperm malformation test, five groups of 10 mice were administered 0, 1, 250, 2,500 or 5,000 mg NaHA/kg bw *via* oral gavage daily for 5 days. Mice were sacrificed 30 days after the final administration, and the malformation rate was calculated. No increase in the number of malformed sperm was observed in any test group when compared to untreated animals.

A safety assessment of HA, potassium hyaluronate and NaHA by the Cosmetic Ingredient Review Expert Panel provided the details of several Japanese genotoxicity studies (Becker et al., 2009). Bacterial reverse mutagenicity testing of NaHA produced by bacterial fermentation (SL-1010) showed no genotoxic activity detectable in *Salmonella* strains TA98, TA102, TA1535 or TA1537 and *E. coli* (WP2*uvr*A) ±S9 activation at concentrations of 31.5 μg/plate, 62.5 μg/plate, 125 μg/plate, 250 μg/plate, 500 μg/plate or 1 mg/plate (Sugiyama and Yagame, 1991). In another bacterial reverse mutagenicity study of NaHA (MW 2000 kDa), no genotoxic activity was detectable in *Salmonella* strains TA98, TA100, TA1535 or TA1537 and *E. coli* (WP2*uvr*A) ±S9 activation at concentrations of 31.5 μg/plate, 62.5 μg/plate, 125 μg/plate, 250 μg/plate, 500 μg/plate or 1 mg/plate (Onishi et al., 1992). Mouse micronucleus testing of NaHA (MW 2400 kDa) was performed at concentrations of 75, 150 or 300 mg/kg (Aruga et al., 1992). No differences were found in the number of PCEs with micronuclei between the control and treatment groups. In a separate study to test NaHA (NRD101; MW 1900 kDa), a bacterial reverse mutation assay, an *in vitro* chromosomal aberration test using Chinese hamster lung fibroblasts and a micronucleus assay in mice were performed (Aruga et al., 1994). No differences in the number of reverse mutation colonies between any test culture and the negative control were observed in *Salmonella* strains TA98, TA100, TA1535 or TA1537 and *E. coli* (WP2*uvr*A) ±S9 activation at concentrations of 312.5 μg/plate, 625 μg/plate, 1250 μg/plate, 2500 μg/plate or 5000 μg/plate. No chromosomal abnormalities were observed in Chinese hamster lung fibroblasts at concentrations of 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml or 1000 μg/ml when treated for 24 or 48 h ±S9 activation. Mouse micronucleus testing of NaHA was performed at

concentrations of 90, 180 or 360 mg/kg either once per day or for 4 consecutive days. No differences were found in the number of PCEs with micronuclei between the control and any treatment groups.

Bloomage sponsored genotoxicity testing of NaHA conducted by the Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention that included a bacterial reverse mutagenicity test, micronucleus test in bone marrow and sperm malformation test (Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b). The reverse mutagenicity test showed no mutagenicity of NaHA in *Salmonella* strains TA97, TA98, TA100 or TA102 ± S9 activation at concentrations of 0.2 mg/plate, 0.5 mg/plate, 1.0 mg/plate, 2.5 mg/plate or 5 mg/plate. A micronucleus test in bone marrow polychromatic erythrocytes (PCE) and a sperm malformation test were performed in mice with 3 doses of NaHA: 440, 880 and 1760 mg/kg bw. Percentages of micronucleated PCEs in all treatment and control groups were not significantly different. Sperm malformation rates in all NaHA dose groups were not statistically different from the control group.

Bloomage sponsored bacterial reverse mutagenicity testing of NaHA conducted by the Consumer Product Testing Company (Consumer Product Testing Company, 2017). No genotoxic activity was detectable in *Salmonella* strains TA97a, TA98, TA100, TA102 or TA1535 ±S9 activation at concentrations of 10 μg/plate, 50 μg/plate, 100 μg/plate, 500 μg/plate or 1 mg/plate.

Rooster comb or cartilage extracts

Bioiberica's RCE (IB0004) that is 60-80% HA (MW 800 kDa) did not exhibit mutagenic activity in *Salmonella* strains TA98, TA100, TA1535 or TA1537 and in *E. coli* WP2 *uvr*A pKM101 ±S9 activation at a concentration of 5 mg/plate (Canut et al., 2012).

The EFSA review of RCE for use as a food ingredient evaluated the available genotoxicity and mutagenicity studies (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013). The panel cited the study by Canut (2012), which did not show mutagenic activity of RCE in tests for gene mutations in bacteria at the highest tested dose of 5 mg/plate ±S9 metabolic activation. The EFSA panel concluded that "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."

A review article described genotoxicity studies of a high molecular weight pharmaceutical-grade NaHA extracted from rooster combs (1000-2900 kDa, Orthovisc®) that is indicated in the treatment of knee pain in osteoarthritis patients (Necas et al., 2008). Orthovisc® was not genotoxic in an *in vitro* sister chromatid exchange assay or a chromosomal aberration assay (both in Chinese hamster ovary cells) nor was it mutagenic in *Salmonella* strains typically used in the Ames assay.

In vitro study details are presented in Table 8.

Table 8. Summary of *In Vitro* Studies for NaHA and HA

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Bacterial reverse mutation assays		
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA97a, TA98, TA100 & TA102 **Assays:** +/- S9 activation **Dose:** 0.008, 0.04, 0.2, 1.0 & 5.0 mg NaHA/plate (fermentation)	**Results and Significance:** • No detectable genotoxic activity at the highest concentration tested, 5 mg NaHA/plate +/- S9 activation	(Zuo et al., 2008)
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA98, TA102, TA1535 & TA1537 and *E. coli* (WP2*uvr*A) **Assays:** +/- S9 activation **Dose:** 31.5, 62.5, 125, 250, 500 & 1000 μg NaHA/plate (fermentation; SL-1010)	**Results and Significance:** • No genotoxic activity detectable at the highest concentration tested, 1 mg NaHA/plate +/- S9 activation	(Sugiyama and Yagame, 1991)
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA98, TA100, TA1535 & TA1537 and *E. coli* (WP2*uvr*A) **Assays:** +/- S9 activation **Dose:** 31.5, 62.5, 125, 250, 500 or 1000 μg NaHA/plate (fermentation; MW 2000 kDa)	**Results and Significance:** • No genotoxic activity detectable at the highest concentration tested, 1 mg NaHA/plate +/- S9 activation	(Onishi et al., 1992)
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA98, TA100, TA1535 & TA1537 and *E. coli* (WP2*uvr*A) **Assays:** +/- S9 activation **Dose:** 312.5, 625, 1250, 2500 or 5000 μg NaHA/plate (fermentation; MW=1900 kDa)	**Results and Significance:** • No significant difference in the number of reverse mutation colonies between the negative control and the highest concentration tested, 5 mg NaHA/plate	(Aruga et al., 1994)
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA98, TA100, TA1535 & TA1537 and *E. coli* WP2*uvr*A pKM101 **Assays:** +/- S9 activation **Dose:** 5.0 mg RCE/plate (IB0004; 60-80% HA; MW=800 kDa)	**Results and Significance:** • RCE did not exhibit mutagenic activity at the concentration tested, 5 mg RCE/plate • EFSA panel cited the results of this study in their conclusion that they had "no safety concerns related to genotoxicity (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013)" of Bioiberica's RCE	(Canut et al., 2012)
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA97, TA98, TA100 & TA102 **Assays:** +/- S9 activation **Dose:** 0.2, 0.5, 1.0, 2.5 & 5.0 mg NaHA/plate dissolved in H_2O (fermentation)	**Results and Significance:** • No increase in revertant colonies in any test strain +/- S9 at the highest concentration tested, 5 mg NaHA/plate • Suggests a lack of mutagenic activity	(Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b); unpublished
Study Design: Bacterial reverse mutation assay **Cell Type:** *S. typhimurium* strains TA97a, TA98, TA100, TA102 &TA1535 **Assays:** +/- S9 activation	**Results and Significance:** • No detectable genotoxic activity at 1 mg NaHA/plate +/- S9 activation	(Consumer Product Testing Company, 2017); unpublished

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Dose: 0.01, 0.05, 0.1, 0.5 & 1.0 mg NaHA/plate dissolved in DMSO (fermentation)		
Mouse micronucleus assays		
Study Design: Mouse micronucleus testing **Cell Type:** Bone marrow polychromatic erythrocytes (PCE) **Animals:** KS mice (5M/5F per group) **Dose:** 1250, 2500 & 5000 mg/kg bw NaHA by gavage. Cyclophosphamide was positive control/corn germ oil negative control	**Results and Significance:** • Percentages of micronucleated PCEs in treatment & control groups were not significantly different • Results suggest that NaHA is not mutagenic to somatic chromosomes of mice up to 5000 mg NaHA/kg bw	(Zuo et al., 2008)
Study Design: Mouse micronucleus testing **Cell Type:** Bone marrow polychromatic erythrocytes (PCE) **Animals:** Mice **Dose:** 75, 150 & 300 mg NaHA/kg bw by gavage (MW= 2400 kDa)	**Results and Significance:** • No differences were found in the number of PCEs with micronuclei between the control and treatment groups up to 300 mg NaHA/kg bw	(Aruga et al., 1992)
Study Design: Mouse micronucleus testing **Cell Type:** Bone marrow polychromatic erythrocytes (PCE) **Animals:** Mice **Dose:** 90, 180 & 360 mg NaHA/kg bw either 1x or for 4 consecutive days by gavage (MW=1900 kDa)	**Results and Significance:** • No differences were found in the number of PCEs with micronuclei between the control and treatment groups up to 360 mg NaHA/kg bw	(Aruga et al., 1994)
Study Design: Mouse micronucleus testing **Cell Type:** Bone marrow polychromatic erythrocytes (PCE) **Animals:** KS mice (5M/5F per group) **Dose:** 440, 880 & 1760 mg NaHA/kg bw by gavage. Cyclophosphamide was positive control/distilled H_2O negative control	**Results and Significance:** • Percentages of micronucleated PCEs in treatment & control groups were not significantly different • Results suggest that NaHA is not mutagenic to somatic chromosomes of mice up to 1760 mg NaHA/kg bw	(Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b); unpublished
Sperm malformation test		
Study Design: Sperm malformation test **Cell Type:** Sperm **Animals:** KS mice (10M per group) **Dose:** 1250, 2500 & 5000 mg NaHA/kg bw by gavage 1x per day for 5 days. Cyclophosphamide was positive control/corn germ oil negative control	**Results and Significance:** • Test group had no significant differences compared to the negative control in the sperm malformation rate of mice, but had significant differences compared to the positive control. • Results suggest that NaHA has no mutagenic toxicity to germ cells of mice up to 5000 mg NaHA/kg bw	(Zuo et al., 2008)
Study Design: Sperm malformation test **Cell Type:** Sperm **Animals:** Mice (10M per group) **Dose:** 440, 880 & 1760 mg NaHA/kg bw in H_2O by gavage 1x per day for 5 days.	**Results and Significance:** • Sperm malformation rates in all NaHA dose groups were not statistically different from the negative control group.	(Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b); unpublished

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Cyclophosphamide was positive control/distilled H_2O negative control	• Results suggest that NaHA has no mutagenic toxicity to germ cells of mice up to 1760 mg NaHA/kg bw	
Chromosomal aberration & sister chromatid exchange		
Study Design: *In vitro* chromosomal aberration assay **Cell Type:** Chinese hamster lung fibroblasts **Assays:** +/- S9 activation **Dose:** 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml or 1000 μg NaHA/ml (MW=1900 kDa)	**Results and Significance:** • No chromosomal abnormalities observed up to 1 mg NaHA/ml +/-S9 activation	(Aruga et al., 1994)
Study Design: *In vitro* chromosomal aberration & sister chromatid exchange assays **Cell Type:** Chinese hamster ovary cells **Dose:** NaHA extracted from rooster combs (Orthovisc®; MW=1000-2900 kDa); dose=NS	**Results and Significance:** • No genotoxicity in *in vitro* chromosomal aberration or sister chromatid exchange assays	(Necas et al., 2008)

DMSO – dimethylsulfoxide; NS – not specified; PCE – polychromatic erythrocytes

Based on the above studies, it can be concluded that neither HA nor NaHA are genotoxic under a variety of accepted tests.

4. Animal Safety Studies with NaHA and HA

a. Acute Studies

Four published (Zuo et al., 2008; Morita, 1991; Wakisaka et al., 1991; Akasaka et al., 1988) and 3 unpublished (Shandong Center for Disease Control and Prevention, 2004; Bloomage Freda Biopharm; Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b) acute toxicity studies in mice and rats were identified that evaluated the oral intake of NaHA produced by bacterial fermentation. Two additional acute toxicity studies in rats were also identified that evaluated the oral intake of RCE or chicken sternal cartilage extract containing 10% HA (Schauss et al., 2007) and 60-80% NaHA (Canut et al., 2012).

Sodium hyaluronate

An oral acute toxicity study in mice was performed as part of a comprehensive study of NaHA (Zuo et al., 2008). A total of 10 male and 10 female mice received an oral gavage dose of 15,000 mg NaHA/kg bw. The molecular weight of the NaHA was not reported; NaHA was provided by a company in Liuzhou City, Guangxi Province, China. The mice were observed for mortality and clinical signs for 14 days. There were no obvious toxic symptoms and no deaths during the 14-day period. The authors concluded that the acute NOAEL for NaHA in mice >15,000 mg/kg bw.

An acute toxicity study was conducted using SL-1010, a NaHA produced by bacterial fermentation, in rats and dogs (Morita, 1991). A single administration in both male and female rats was given at dose levels of 200 mg/kg bw for the oral route and 300 mg/kg for the subcutaneous route. In dogs, a single dose of 50 mg/kg bw was given subcutaneously. No signs of toxicity were observed for 14 days post-administration. No mortality was observed from either the oral or subcutaneous administration of SL-

1010, and no macroscopic major organ or body weight changes were attributable to SL-1010 dosing in rats and dogs by either route. The LD_{50} value was estimated to exceed 200 mg/kg bw for the oral route for rats and was >300 mg/kg bw for the subcutaneous route in both sexes of rats; it was also >50 mg/kg bw for the subcutaneous route in both sexes of dogs.

Another acute study of SL-1010 was conducted in ICR mice at an oral dose of 500 mg/kg bw, as well as, subcutaneous and intraperitoneal administrations of 600 mg/kg bw (Wakisaka et al., 1991). SL-1010 did not cause any toxic signs or animal deaths by any route of administration. In each sex, the oral LD_{50} was >500 mg/kg bw, and the subcutaneous and intraperitoneal LD_{50} was >600 mg/kg bw.

No mortalities were reported in an acute oral toxicity study of ICR mice after a single dose of HA (produced by fermentation using *Streptococcus zooepidemicus*) >1,200 mg/kg bw (Akasaka et al., 1988).

Bloomage contracted with Shandong Sanitation and Antiepidemic Station to perform an acute toxicity study of NaHA in Kunming mice (10M/10F; unpublished) (Shandong Center for Disease Control and Prevention, 2004). The animals were given an intragastric dose of 2000 mg/kg bw NaHA. The molecular weight of the NaHA was not reported. Animals were observed for 14 days after dosing. No deaths or toxic symptoms were found in any of the animals. The investigators assigned a LD_{50} for NaHA in mice of >2000 mg NaHA/kg bw.

Bloomage contracted with the Shandong Sanitation and Antiepidemic Station to perform an acute oral toxicity study of a range of NaHA doses using Kunming mice (20M/20F; unpublished) (Bloomage Freda Biopharm). The animals were given intragastric doses of 1000, 2150, 4640 or 10,000 mg/kg bw, respectively. The molecular weight of the NaHA was not reported. Animals were observed for 14 days after one-time gavage. Food consumption of all of the animals was normal and no deaths or toxic symptoms were observed for any of the animals. The investigators assigned a LD_{50} for NaHA in mice of >10,000 mg/kg bw.

Bloomage also conducted an acute toxicity study of NaHA in Wistar rats (10M/10F; unpublished) with the study executed through the Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention by using an intragastric dose of 5280 mg/kg bw (Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b). The molecular weight of the NaHA was 270 kDa. After the one-time administration, no obvious toxic symptoms and no deaths were recorded during the 14 days of observation. It was concluded that the maximum tolerated dose of NaHA for oral acute toxicity for rats is >5280 mg/kg bw.

In all of the acute toxicity studies described above, no significant adverse effects were observed that could be attributed to the test article. Therefore, it can be concluded that the NOAEL was the highest dose administered.

Rooster comb or cartilage extracts

The acute toxicity of RCE that contains high levels of NaHA was evaluated (Canut et al., 2012). The test article, IB0004 (Bioiberica S.A., Barcelona, Spain) contains 60-80% NaHA, 20% glycosaminoglycans and 20% partially hydrolyzed proteins with a molecular weight of about 800 kDa. Sprague-Dawley rats (5M/5F) were administered a single oral gavage dose of 2000 mg/kg bw and were observed for 14 days. Due to the viscosity and density of the RCE and the need for high dosing volumes, the dose was divided into two parts each 1000mg/kg bw, with 4 h between the first and

second administration. No mortality or changes in weight were observed nor were other adverse effects noted during the observation period. Therefore, the LD_{50} for rats by the oral route was determined to be >2000 mg/kg bw.

Schauss et al. conducted an acute toxicity study in rats to evaluate the safety of a preparation of chicken sternal cartilage (BioCell Collagen II®) containing collagen type II (60%), chondroitin sulfate (20%), HA (10%), and other proteoglycans (1%) (Schauss et al., 2007). Sprague-Dawley rats (5M/5F) were administered a single oral dose of 5000 mg/kg bw and observed for 14 days. All animals survived and exhibited normal body weight gain throughout the study. A gross necroscopic examination conducted on day 15 revealed no gross pathological lesions in any of the animals.

Study details are presented in Table 9. Overall, the unpublished acute toxicity animal studies corroborate that Bloomage's NaHA manufacturing process and extended molecular weight range do not change the safety profile of the material.

b. Repeated Oral Dose Studies

Two published 90-day feeding studies were identified in the literature (Zuo et al., 2008; Ishihara et al., 1996). Both 30-day and 90-day unpublished repeated dose oral toxicity studies of NaHA produced by bacterial fermentation were provided by Bloomage (Shandong Center for Disease Control and Prevention, 2004; Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b). Three additional published repeated-dose oral studies of RCE or chicken sternal cartilage extract containing 10% HA (Schauss et al., 2007) and 60-80% NaHA (Canut et al., 2012; Bioiberica, 2010) were identified. No chronic studies exceeding 90-days were identified.

Sodium hyaluronate

A 90-day feeding study of NaHA by gavage in SD rats was performed as part of a safety study (Zuo et al., 2008). Ninety-six rats were randomly divided into 4 groups (12M/12F) with NaHA dose levels of 0, 667, 1000 or 1333 mg NaHA/kg bw/day. NaHA was provided by a company in Liuzhou City, Guangxi Province, China. The MW range of the NaHA was not provided. No body weight or food consumption changes were observed between test and control animals. At termination, there was no difference in the hematological parameters measured (*i.e.*, red blood cell and white blood cell counts) or in the clinical biochemistry parameters measured (*i.e.*, AST, ALT, BUN, creatinine, cholesterol, triglycerides, total protein, albumin or glucose). There were no significant differences in the absolute or relative weights of liver, kidney, spleen or testes between the test and control animals. At necropsy, there were no macroscopic findings in the liver, kidney, stomach, spleen, testicle or ovary, and no histopathological alterations were observed for any of these organs. The authors of the study suggest that the study supports a NOAEL of 1333 mg NaHA/kg bw/day.

In another 90-day oral toxicity study, NaHA was administered by oral gavage at doses of 0, 3, 12, or 48 mg/kg bw/day to SD rats (10M/10F per group) (Ishihara et al., 1996). Additional satellite recovery groups (5M/5F per group) receiving the control or high dose were observed for a 28-day period following the discontinuation of NaHA administration. Animals were assessed regularly for general signs (not further specified), body weight, food consumption and water consumption, and ophthalmoscopic examinations were conducted at Week 13 and at the end of the recovery period. Fecal samples were collected at Weeks 0 and 7 and prior to necropsy to determine the viable count of bacteria in feces (including *Bacteroides* culture) and urine samples were collected at Week 13 and at the end of the recovery period for urinalysis (including pH, protein, glucose, ketone body,

urobilinogen, bilirubin, occult blood, sediments, color and volume). At sacrifice, blood samples were collected for hematological (including erythrocyte count, leukocyte count, hematocrit, hemoglobin, platelet count, mean cell hemoglobin, mean cell volume, mean cell hemoglobin concentration, reticulocyte count and prothrombin time) and blood biochemistry (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactic dehydrogenase, creatinine phosphokinase, glucose, total cholesterol, triglyceride, phospholipid, total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, inorganic phosphate, calcium, sodium, potassium, chloride, albumin globulin ratio, testosterone (males), progesterone (females), corticosterone, aldosterone, follicle-stimulating hormone, luteinizing hormone and adrenocorticotrophic hormone) analyses, and organs [brain, hypophysis, submaxillary gland, thyroid and parathyroids, heart, thymus, spleen, lungs, liver, kidneys, adrenals, cecum, small intestine, testes, epididymides, prostate, seminal vesicle, ovaries, uterus and fat around the epididymides (males) and uterus (females)] were collected for weighing. Histological examination of these organs, as well as the trachea, pancreas, tongue, esophagus, stomach, duodenum, jejunum, ileum, colon, rectum, mesenteric lymph node, urinary bladder, vagina, mammary gland (female), spinal cord, thigh muscle and sternum was conducted. No significant differences in body weight, general signs, ophthalmology, histopathology, or hematological parameters were observed between groups. Although sporadic, non-dose dependent and statistically significant differences were reported in food efficiency (increased in low-dose females, and mid- and high-dose males), water consumption (increased in high-dose females), the viable count of *Bacteroides* in the feces (increased in high-dose males and females), blood biochemistry (decrease in total protein in low- and mid-dose males, a decrease in calcium in mid- and high-dose males, and an increase in blood urea nitrogen in high-dose males), urinalysis (increase in urine volume and decrease in specific gravity in high-dose males after the recovery period; decreases in sodium, potassium, and chloride in low- and mid-dose males after the treatment period and high-dose males at the end of the recovery period; and an increase in potassium in low-dose females after the treatment period), and absolute and relative organ weights (including a decrease in hypophysis weight in low- and high-dose males; increase in heart weight of mid- and high-dose males; increase in liver weight of mid-dose males; decrease in liver weight of low-dose females; increase in cecum weight in low- and high-dose males; and a tendency toward an increase in cecum weight in mid-dose males; observations were not accompanied by histopathological changes) in the various dose-groups compared to controls, due to the absence of findings in both sexes and/or the lack of a dose-response relationship, and the absence of histological alterations in the liver and kidneys, the observed differences were deemed not to be toxicologically relevant. The quality of the study supports a NOAEL of 48 mg NaHA/kg bw/day, the highest dose tested .

Bloomage sponsored a 30-day oral toxicity study of NaHA in rats (unpublished) that was conducted by the Shandong Center for Disease Control and Prevention (Shandong Center for Disease Control and Prevention, 2004). Eighty Wistar rats (40M/40F) were randomly divided into 4 groups. The NaHA dose levels were 0, 167, 500 or 1500 mg/kg bw with the NaHA added to the basic feed for 30 days. The MW range of the NaHA was not provided. No differences in body weight gain or the food utilization rate were observed between the test and control animals. Routine blood tests (*i.e.*, hemoglobin, erythrocyte counting, total leukocyte counting and differential leukocyte counting) and blood biochemistry tests (*i.e.*, BUN, creatinine, cholesterol, triglyceride, blood glucose, total protein, albumin, glutamic-pyruvic transaminase and glutamic-oxalacetic transaminase) of the animals were all in the normal range. Pathological examination of the visceral organs did not show any significant abnormalities. Weight and organ coefficients of the liver, spleen, kidney and testicle showed no significant differences between the test and control groups. The authors of the study concluded that the conditions of the study support a NOAEL of 1500 mg NaHA/kg bw/day.

Bloomage sponsored a 90-day oral toxicity study of NaHA in rats (unpublished) by the Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention (Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b). Eighty Wistar rats (40M/40F) were randomly divided into 4 groups (10/sex/group) with NaHA dose levels of 0, 330, 670 or 1000 mg/kg bw. The MW of the NaHA was 270 kDa and was added to the basic feed for 90 days. During the course of the study, no physical or behavioral changes were observed in any of the test animals. No significant differences in body weight or food utilization rates were noted between the test and control groups ($P>0.05$). Routine blood tests (*i.e.*, hemoglobin, erythrocyte counting, total leukocyte counting and differential leukocyte counting) and blood biochemistry tests (*i.e.*, BUN, creatinine, cholesterol, triglyceride, blood glucose, total protein, albumin, glutamic-pyruvic transaminase and glutamic-oxalacetic transaminase) of the animals were all in the normal range. Organ weights and organ-body ratios for liver spleen, kidney and testicle measured during gross necropsy showed no significant differences ($P>0.05$) except for a statistically significant decrease in relative but not absolute spleen weight in the 1000 mg/kg bw females. Histopathologic examination revealed no pathological changes of the liver, kidney, spleen, stomach, duodenum, testes or ovaries. The authors of the study concluded that the conditions of the study support a NOAEL of 1000 mg NaHA/kg bw/day.

No adverse effects were observed in the subchronic studies that could be attributed to the test article. Therefore, the NOAEL was reported to be the highest dose administered.

Rooster comb or cartilage extracts

A 4-week oral toxicity study of Bioiberica's RCE (IB0004; 60-80% NaHA) was conducted using Wistar rats administered doses of 0 (15M/15F), 5 (10M/10F), 55 (10M/10F) or 600 (15M/15F) mg/kg bw/day by oral gavage for 4 weeks (Bioiberica, 2010). Once treatment was complete, 5 males and 5 females each from the control and high-dose group received a standard rodent diet for 2 weeks (*i.e*., during the recovery period). Rats were assessed for mortality, body weight, and food and water intake. Ophthalmoscopic observations, hematology (including erythrocyte count, hemoglobin, hematocrit, platelet count, differential leukocyte counts, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, leucocytes, platelet count, prothrombin time and activated partial thromboplastin time), blood biochemistry (including glucose, urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, cholesterol, triglycerides, inorganic phosphates, total protein, calcium, albumin, sodium, potassium and chlorine), and urinalysis (including color, volume, pH, specific gravity, protein, glucose, bilirubin, urobilinogen, ketones , albumin, globulin and albumin/globulin ratio) were conducted during the last week of treatment, and the last week of the recovery period. Organ weights were determined, and gross and histopathological examinations of organs (including the adrenal glands, aorta, brain, eyes and optic nerves, femur, heart, intestines, kidneys and ureters, larynx, liver, lungs, lymph nodes, mammary area, esophagus, ovaries, pancreas, Peyer's patches, pituitary, prostate, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes and epididymides, thymus, thyroid and parathyroid, tissue masses or tumors, tongue, trachea, urinary bladder, uterus and vagina) were conducted upon sacrifice. One female receiving the mid dose, and 2 males and 1 female receiving the high dose died during the study. The cause of death in 2 males from the high-dose group was attributed to misgavage; however, the cause of death in the females could not be established. The males that died during the study due to misgavage exhibited a foamy red content in the trachea, enlarged reddish-colored lungs and red colored mandibular lymph nodes, whereas the females that died exhibited a reddish liquid content in the jejunum and blackish

mesenteric lymph nodes. None of the deaths were attributed to RCE. No clinical signs were attributed to the test article, and no differences in body weight, food intake, ophthalmoscopic observations, urinalysis, or gross or histopathological examinations were noted between groups receiving RCE and the control group. A significant non-dose dependent reduction in calcium levels was observed in all groups receiving RCE at Week 4 compared to the control group; however, the calcium levels observed were within the normal range. In addition, calcium levels were not significantly different between animals administered 600 mg RCE/kg body weight/day and controls after the 14-day recovery period. Sporadic, non-dose dependent significant differences were observed between controls and animals receiving RCE for some hematological parameters [decreased hemoglobin in mid-dose females, increased mean corpuscular hemoglobin concentration (MCHC) and activated partial thromboplastin time in high-dose females, decreased MCHC in mid-dose males, and decreased segmented neutrophils in low- and mid- dose males]; however, there were no significant differences between animals administered RCE and controls in hematological parameters following the 14-day recovery period. Significantly lower relative thymus weight was reported in high-dose females sacrificed after the 4-week exposure period compared to controls, although this was not accompanied by histopathological changes and was not observed in females sacrificed after the 14-day recovery period. Therefore, this was considered not to be of toxicological relevance. Animals that received the high dose and were sacrificed after the recovery period were reported to have statistically significant differences in certain absolute organ weights (including decreased weights of the thymus, spleen, and liver in males; and increased weights of the uterus and thyroids/parathyroids in females) compared to controls; however, the authors did not attribute these variations to the test compound due to the absence of differences from controls in animals sacrificed immediately after the treatment period. The authors concluded that under the conditions of this study, the NOAEL was 600 mg/kg bw/day, the highest dose tested (which provides 360 mg NaHA/kg bw/day).

A subchronic toxicity study of Bioiberica's RCE (IB0004; 60-80% HA) was conducted using Wistar rats administered doses of 0 (15M/15F), 5 (10M/10F), 55 (10M/10F) or 600 (15M/15F) mg/kg bw/day by oral gavage for 13 weeks (Canut et al., 2012). The study was conducted in compliance with OECD Principles of Good Laboratory Practices and Directive 2004/10/EC of the European Parliament and the Council of 11 February 2004. The study procedures also met or exceeded guidelines for repeated dose toxicity studies[7]. Once treatment was complete, 5 males and 5 females each from the control and high-dose group received a standard rodent diet for 4 weeks (*i.e.*, during the recovery period). The highest dose of 600 mg/kg body weight/day was selected due to the limited solubility of the test compound in water. Animals were assessed regularly for mortality, clinical signs, body weight, and food consumption, and ophthalmoscopic examinations were performed at Weeks 0 and 13 and at the end of the 4-week recovery period. Blood and urine samples were collected for hematological (including erythrocyte, hemoglobin, hematocrit, hemoglobin concentration distribution width, mean corpuscular volume, red cell volume distribution width, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, reticulocyte count, reticulocyte maturity index, total leukocyte count, differential leukocyte count, prothrombin time and activated partial thromboplastin time) and biochemical (including glucose, urea, creatinine, total bilirubin, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine kinase, y-glutamyl transferase, calcium, inorganic phosphorus, sodium,

[7] Annex IV A Part B. Method B. 26. Subchronic oral toxicity test: 90-day repeated oral dose using rodent species. Note for Guidance on Repeated Dose Toxicity. CPMP/SWP/1042/99. 27 July 2000 and ICH Topic S4A. Note for Guidance on Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing) (CPMP/ ICH/300/95).

potassium, chloride, total protein, albumin, globulin and albumin/globulin ratio) analyses and urinalysis (including specific gravity, volume, color, appearance, pH, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, erythrocytes, leukocytes and sediment) at Week 13 of the study. At terminal sacrifice, organ weights were determined and tissue samples of the adrenal glands, aorta, bone marrow, brain, epididymides, esophagus, eyes with optic nerve, femur, heart, intestines, kidneys, larynx, liver, lungs, lymph nodes, mammary gland area, ovaries, pancreas, pituitary gland, prostate and seminal vesicles, salivary glands, sciatic nerve, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid, trachea, urinary bladder, uterus, vagina and all gross lesions were collected for gross and histopathological examination. No compound-related mortality, and no significant differences in food consumption, ophthalmoscopic parameters, blood biochemistry values, urinalysis or gross and histopathological observations were reported compared to controls. In males, body weight and body weight gain was not significantly different from controls during the treatment and recovery period. Body weight gain in mid-dose females was statistically higher on Days 22, 64, 71, 75 and 82 of the study; however, body weight and body weight gain were not different in the high-dose group during the treatment period or in any animals included in the recovery group compared to controls. Thus, the authors considered the differences in body weight to be of no toxicological relevance as they were transient, minor in magnitude, and not dose-dependent. Statistically significant increases were reported in mean corpuscular hemoglobin index and reticulocyte count in males of the low-dose group, mean corpuscular volume in males of the mid-dose group, and platelet values in females in the high-dose group; however, these effects were determined to be of no toxicological significance due to the lack of a dose-response relationship and the absence of findings in the opposite sex. Furthermore, no differences were observed in any hematological parameter in high dose animals sacrificed after the 4-week recovery period compared to controls. Relative liver (to body) weight was significantly higher in low and high-dose females compared to controls, although this effect was not observed in male animals. Thus, the authors considered the increases in relative liver to body weights to be of no biological significance due to the lack of effects in males, the absence of histopathological correlates, and the lack of effects on biochemical markers of liver function. No other differences in organ weights were reported. Under the conditions of this study, the authors determined the NOAEL to be 600 mg RCE/kg bw/day, the highest dose tested (which provides 360 mg NaHA/kg bw/day).

A 90-day subchronic oral toxicity study was conducted to evaluate the safety of hydrolyzed chicken sternal cartilage (BioCell Collagen II®, BioCell Technology, LLC, Anaheim, CA) containing collagen type II (60%), chondroitin sulfate (20%) and hyaluronic acid (10%) (Schauss et al., 2007). The MW of the test product components were not specified. Sprague-Dawley rats (40M/40F) were administered 0, 30, 300 or 1000 mg/kg bw/day of the test product by oral gavage for 90 days. Animals were regularly assessed for body weight, food and water consumption and clinical signs (including changes in skin, fur, eyes and mucous membranes, occurrence of secretions and excretions, autonomic activity, changes in gait, posture, response to handling, presence of clonic or tonic movements, sterotypes or bizarre behavior). At baseline and on Day 79, animals underwent ophthalmoscopic examination. Blood samples were collected on Day 86 and assessed for hematological parameters (including hematocrit, hemoglobin concentration, erythrocyte count, total white blood cell count, differential leukocyte count, platelet count, absolute reticulocyte count, mean corpuscular hemoglobin, red cell distribution width, mean corpuscular volume, prothrombin time and activated partial thromboplastin time); blood biochemistry (including albumin, alkaline phosphatase, blood creatinine, calcium, chloride, globulin, glucose, inorganic phosphorus, potassium, alanine aminotransferase, aspartate aminotransferase, sodium, sorbitol dehydrogenase, total bilirubin, total cholesterol, total protein, triglycerides and blood urea nitrogen); and serology (including sendai virus,

sialodacryloadenitis virus, Toolan's H-1, Pasteurella multocida, Parvovirus non-structural 1, Pneumonia virus of mice, Kilham's rat virus, Reovirus Type 3, and Rat Parvovirus). At sacrifice, organs were examined for macroscopic observations (including external surfaces and orifices, and cranial, thoracic, and abdominal cavities and their contents) and histopathology [including all gross lesions, lungs, trachea, brain (including sections of the medulla/pons, cerebellar cortex and cerebral cortex), spinal cord (three levels: cervical, mid-thoracic and lumbar), eyes, pituitary, thyroid/parathyroid, thymus, heart, aorta, sternum with bone marrow, liver, spleen, kidneys, pancreas, adrenals, ovaries, testes, uterus, vagina, accessory genital organs (epididymides, prostate, and seminal vesicles), female mammary gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, representative lymph node (mesenteric and mandibular), salivary glands, peripheral nerve (sciatic) and skin]. Organ weights were determined for the liver, kidneys, adrenals, brain, heart, thymus, spleen, uterus, ovaries, testes and epididymides. No adverse effects or clinical signs of toxicity attributed to the test compound were reported. No differences in food consumption, ophthalmoscopic examinations or hematology were observed in treated rats compared to controls. Serology results were not reported. Sporadic, non-dose dependent and statistically significant differences from control animals were reported for body weight gain (both increased and decreased in certain treatment groups at various timepoints compared to controls) and some blood biochemical parameters (including a decrease in alkaline phosphatase activity in high dose males, an increase in albumin in mid-dose males, and an increase in globulin in high dose females); however, the authors deemed these to be non-adverse and unrelated to treatment due to the lack of a dose-response relationship. A statistically significant increase in absolute brain weights in low-dose females and a decrease in relative (to brain) spleen weights in mid-dose males compared to controls were reported; however, these effects were not reported in high-dose animals and were not accompanied by histopathological changes. Therefore, the authors determined these findings to be non-adverse and unrelated to treatment. The authors reported no compound-related changes following gross or histopathological examinations. Based on the results of the study, a NOAEL of 1,000 mg BioCell Collagen II (providing 100 mg HA)/kg body weight/day, the highest dose tested, can be reasonably supported.

The details of repeated-dose animal studies can be found in Table 9. Overall, the unpublished repeated dose animal studies corroborate that Bloomage's NaHA manufacturing process and extended molecular weight range do not change the safety profile of the material.

Table 9. Summary of Animal Safety Studies for NaHA and HA

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Acute Studies		
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** 20 Kunming mice (10M/10F) **Dose/Delivery/Vehicle/Frequency:** Single dose by oral gavage of 15,000 mg/kg bw NaHA in corn germ oil (MW=NS)	**Outcome Measurements:** • Mortality & clinical signs **Results and Significance:** • No obvious toxic symptoms & no deaths • LD_{50}: >15,000 mg NaHA/kg bw	(Zuo et al., 2008)
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** Rat (M/F; animal numbers NS)	**Outcome Measurements:** • Mortality & signs of toxicity • Body weight determination	(Morita, 1991)

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Dose/Delivery/Vehicle/Frequency: Single oral dose of 200 mg/kg bw NaHA (SL-1010; fermentation; MW=1800-2100 kDa)	• Macroscopic examination of major organs at necropsy **Results and Significance:** • No signs of toxicity or deaths • No changes in body weight & no macroscopic observations • LD_{50}: >200 mg NaHA/kg bw	
Study Design: Acute oral toxicity **Study Length:** NS **Animals:** ICR mice (animal numbers NS) **Dose/Delivery/Vehicle/Frequency:** Single oral dose of 500 mg/kg bw NaHA (SL-1010; fermentation; MW=1800-2100 kDa)	**Outcome Measurements:** • Mortality & signs of toxicity **Results and Significance:** • LD_{50}: >500 mg NaHA/kg bw	(Wakisaka et al., 1991)
Study Design: Acute oral toxicity **Study Length:** NS **Animals:** ICR mice (animal sex/numbers NS) **Dose/Delivery/Vehicle/Frequency:** Single oral dose of 1,200 mg/kg bw NaHA (fermentation; MW=NS)	**Outcome Measurements:** • Mortality **Results and Significance:** • No deaths • LD_{50}: >1,200 mg NaHA/kg bw	(Akasaka et al., 1988)
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** 20 Kunming mice (10M/10F) **Dose/Delivery/Vehicle/Frequency:** Two doses by oral gavage with a total dose of 2000 mg/kg bw NaHA (fermentation; MW=NS) suspended in peanut oil	**Outcome Measurements:** • Mortality and clinical signs **Results and Significance:** • All animals survived the 14-day observation period • No obvious toxic symptoms & no deaths • LD_{50}**:** >2000 mg NaHA/kg bw	(Shandong Center for Disease Control and Prevention, 2004); unpublished
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** 40 Kunming mice (20M/20F) **Dose/Delivery/Vehicle/Frequency:** Single dose by oral gavage of 1000, 2150, 4640 or 10,000 mg/kg bw NaHA (fermentation; MW=NS)) suspended in peanut oil	**Outcome Measurements:** • Mortality & signs of toxicity **Results and Significance:** • No significant toxic symptoms & no deaths • Food consumption of all animals was normal • LD_{50}: >10,000 mg NaHA/kg bw	(Bloomage Freda Biopharm); unpublished
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** 20 Wistar rats (10M/10F) **Dose/Delivery/Vehicle/Frequency:** Three doses by oral gavage with a total dose of 5280 mg/kg bw NaHA (fermentation; MW=270 kDa) suspended in distilled H_2O	**Outcome Measurements:** • Mortality & clinical signs **Results and Significance:** • No obvious toxic symptoms & no deaths • MTD: >5280 mg NaHA/kg bw	(Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b); unpublished

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** Sprague-Dawley rats (5M/5F) **Dose/Delivery/Vehicle/Frequency:** Two doses by oral gavage separated by 4 h for a total dose of 2000 mg/kg bw RCE (IB0004 Bioiberica SA; 60-80% NaHA; MW=800 kDa) suspended in distilled H_2O	**Outcome Measurements:** • Mortality & signs of toxicity • Body weight determination • Macroscopic examination of major organs & tissues at necropsy **Results and Significance:** • No deaths • Normal body weight gain • Macroscopic & gross pathology revealed no lesions attributable to RCE • LD_{50}: >2000 mg RCE/kg bw	(Canut et al., 2012)
Study Design: Acute oral toxicity **Study Length:** 14 days **Animals:** Sprague-Dawley rats (5M/5F) **Dose/Delivery/Vehicle/Frequency:** Single oral dose of 5000 mg/kg bw chicken sternal cartilage preparation (BioCell Collagen II®; 10% HA; MW=NS) suspended in distilled H_2O	**Outcome Measurements:** • Mortality & signs of toxicity • Body weight determination • Macroscopic examination of major organs & tissues at necropsy **Results and Significance:** • No deaths • Normal body weight gain • Macroscopic & gross pathology revealed no lesions attributable to cartilage preparation • LD_{50}: >5000 mg/kg bw (chicken sternal cartilage preparation)	(Schauss et al., 2007)
Repeated Oral Dose Studies		
Study Design: 90-d feeding study **Study Length:** 90 day **Animals:** 96 SD rats (12M/12F per group) **Dose/Delivery/Vehicle/Frequency:** 1x daily by gavage at 0, 667, 1000 or 1333 mg NaHA/kg bw/day (fermentation; MW=NS) suspended in corn germ oil	**Outcome Measurements:** • Food consumption, animal weight and absolute & relative organ weights (liver, kidney, spleen, testis) • Hematological testing [hemoglobin, red blood cells, white blood cells, platelets & leukocytes (lymphocytes, monocytes, granulocytes)] • Blood biochemistry testing (BUN, creatinine, total cholesterol, triglycerides, total protein, albumin, glucose, aspartate aminotransferase, alanine aminotransferase) • Gross pathology & histopathology of liver, kidney, stomach, intestines, spleen, testicle & ovary at termination of study **Results and Significance:** • No anti-feeding or behavioral changes • No significant differences in body weight & food consumption • No significant differences in hematological parameters	(Zuo et al., 2008)

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	• No significant differences in absolute or relative organ weights (liver, spleen, kidney & testicle) • No macroscopic or histological alternations in the liver, kidney, stomach, intestines, spleen, testicle or ovary • NOAEL=1333 mg NaHA/kg bw/day	
Study Design: 90-day oral toxicity with control & high-dose 28-d recovery groups **Study Length:** 90 days **Animals:** SD rats (10M/10F per group); control & high-dose 28-d recovery groups (5M/5F) **Dose/Delivery/Vehicle/Frequency:** 1x daily by gavage at 0, 3, 12 or 48 mg NaHA/kg bw/day (MW=NS)	**Outcome Measurements:** • Body weight, food efficiency & behavior • Routine blood testing & blood biochemistry tests • Urinalysis • Gross pathology & histopathology of brain, hypophysis, submaxillary gland, thyroid and parathyroids, heart, thymus, spleen, lungs, liver, kidneys, adrenals, cecum, small intestine, testes, epididymides, prostate, seminal vesicle, ovaries, uterus, trachea, pancreas, tongue, esophagus, stomach, duodenum, jejunum, ileum, colon, rectum, mesenteric lymph node, urinary bladder, vagina, mammary gland (female), spinal cord, thigh muscle & sternum **Results and Significance:** • No significant differences in body weight, general signs, ophthalmology, histopathology or hematological parameters were observed between groups • Sporadic, not dose-dependent differences were reported in food efficiency, blood biochemistry, urinalysis and absolute and relative organ weights. Due to absence of findings in both sexes and/or the lack of a dose-response relationship, and the absence of histological alterations in the liver and kidneys, the observed differences were deemed not to be toxicologically relevant. • NOAEL=48 mg NaHA/kg bw/day (low dose based on formulation of product; 1% ophthalmic solution)	(Ishihara et al., 1996)
Study Design: 30-d repeated dose oral toxicity **Study Length:** 30 d **Animals:** 80 Wistar rats (40M/40F) divided into 4 groups **Dose/Delivery/Vehicle/Frequency:** 1x daily added to basic feed at 0, 167, 500 or 1500 mg/kg bw NaHA (fermentation; MW=NS)	**Outcome Measurements:** • Body weight gain & food utilization rate • Routine blood testing: hemoglobin, erythrocyte counting, total leukocyte counting and differential leukocyte counting • Blood biochemistry tests: BUN, creatinine, cholesterol, triglyceride, blood glucose, total protein, albumin, glutamic-pyruvic transaminase & glutamic-oxalacetic transaminase • Macroscopic & gross pathology of major organs & tissues: liver, spleen, stomach, intestines, testicles & ovaries	(Shandong Center for Disease Control and Prevention, 2004); unpublished

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	Results and Significance: • No deaths or signs of toxicity • Body weight, food consumption, food efficiency & weight gain not significantly different between test & control groups • All routine blood test results within normal range • ALT & TG of high & low dose males, TG of middle dose males & TG of high & middle dose females were statistically different from controls ($P<0.05$ or $P<0.01$), but still within the normal range. No significant difference in other blood biochemistry parameters • Weight and organ coefficients of the liver, spleen, kidney and testicle showed no significant differences between the test and control groups • No abnormal findings on necropsy & no treatment-related differences in the histopathology of the liver, spleen, kidney, stomach, intestines, testicles or ovaries • NOAEL=1500 mg NaHA/kg bw/day	
Study Design: 90-d oral toxicity study **Study Length:** 90 d **Animals:** 80 Wistar rats (40M/40F) divided into 4 groups (10 sex/group) **Dose/Delivery/Vehicle/Frequency:** 1x daily added to basic feed at 0, 330, 670 or 1000 mg/kg bw NaHA (fermentation; MW=270 kDa)	**Outcome Measurements:** • Body weight gain & food utilization rate • Routine blood testing: hemoglobin, erythrocyte counting, total leukocyte counting and differential leukocyte counting • Blood biochemistry tests: BUN, creatinine, cholesterol, triglyceride, blood glucose, total protein, albumin, glutamic-pyruvic transaminase and glutamic-oxalacetic transaminase • Macroscopic & gross pathology of major organs & tissues: liver, spleen, stomach, intestines, testicles & ovaries **Results and Significance:** • No deaths or signs of toxicity • Body weight, food consumption, food efficiency & weight gain not significantly different between test & control groups • All routine blood test results within normal range; some sporadic but not dose-dependent differences observed • Blood biochemistry parameters within the normal range; some sporadic but not dose-dependent differences observed • Weight & organ coefficients of the liver, spleen, kidney & testicle showed no significant differences between the test and control groups.	(Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b); unpublished

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	• No abnormal findings on necropsy & no treatment-related differences in the histopathology of the liver, spleen, kidney, stomach, intestines, testicles or ovaries • NOAEL=1000 mg NaHA/kg bw/day	
Study Design: 28-d oral toxicity with control & high-dose 14-d recovery groups **Study Length:** 28 days **Animals:** Wistar rats (15M/15F control & high-dose: 10M/10F all other groups) **Dose/Delivery/Vehicle/Frequency:** 1x daily by gavage at 0, 5, 55 or 600 mg/kg bw/day of RCE (IB0004 Bioiberica SA; 60-80% NaHA; MW=800 kDa) suspended in distilled H_2O	**Outcome Measurements:** • Mortality, body weight & food intake • Clinical signs: changes in skin, fur, eyes & mucous membranes; changes in respiratory, circulatory, autonomic & central nervous systems; somatomotor activity; behavior • Hematological testing (erythrocyte count, hemoglobin, hematocrit, platelet count, differential leukocyte counts, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, leucocytes, platelet count, prothrombin time & activated partial thromboplastin time) & blood biochemistry (glucose, urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, cholesterol, triglycerides, inorganic phosphates, total protein, calcium, albumin, sodium, potassium & chlorine) • Urinalysis (color, volume, pH, specific gravity, protein, glucose, bilirubin, urobilinogen, ketones , albumin, globulin & albumin/globulin ratio) • Full autopsy on all animals: macroscopic examination, organ weight & histological examination (adrenal glands, aorta, brain, eyes and optic nerves, femur, heart, intestines, kidneys and ureters, larynx, liver, lungs, lymph nodes, mammary area, esophagus, ovaries, pancreas, Peyer's patches, pituitary, prostate, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes and epididymides, thymus, thyroid and parathyroid, tissue masses or tumors, tongue, trachea, urinary bladder, uterus & vagina) **Results and Significance:** • One female receiving the mid dose, and 2 males and 1 female receiving the high dose died during the study. The cause of death in 2 males from the high-dose group was attributed to misgavage; however, the cause of death in the female could not be established. None of the deaths were attributed to RCE. • No significant differences in body weight or food intake between groups • A significant non-dose dependent reduction in calcium levels was observed in all groups receiving RCE at	(Bioiberica, 2010)

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	Week 4 compared to the control group; however, the calcium levels observed were within the normal range. Calcium levels were not significantly different between animals administered 600 mg RCE/kg body weight/day and controls after the 14-day recovery period. • No treatment-related differences in hematological or urinary analysis • No treatment-related macroscopic alterations were observed during necropsy. Significantly lower relative thymus weight was reported in 600 mg/kg bw/day females sacrificed after the 4-week exposure period compared to controls, although this was not accompanied by histopathological changes and was not observed in females sacrificed after the 14-day recovery period. • No histological alterations related to RCE intake were reported in any of the animals • NOAEL=600 mg RCE/kg bw/day (360 mg NaHA/kg bw/day)	
Study Design: Subchronic toxicity study with control & high-dose 4-wk recovery groups **Study Length:** 13 wk **Animals:** Wistar rats (15M/15F control & high-dose: 10M/10F all other groups) **Dose/Delivery/Vehicle/Frequency:** 1x daily by gavage at 0, 5, 55 or 600 mg/kg bw/day of RCE (IB0004 Bioiberica SA; 60-80% NaHA; MW=800 kDa) suspended in distilled H_2O	**Outcome Measurements:** • Mortality, body weight & food intake • Clinical signs: changes in skin, fur, eyes & mucous membranes; changes in respiratory, circulatory, autonomic & central nervous systems; somatomotor activity; behavior • Hematological testing (erythrocyte, hemoglobin, hematocrit, hemoglobin concentration distribution width, mean corpuscular volume, red cell volume distribution width, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, reticulocyte count, reticulocyte maturity index, total leukocyte count, differential leukocyte count, prothrombin time & activated partial thromboplastin time) & clinical biochemistry (glucose, urea, creatinine, total bilirubin, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine kinase, y-glutamyl transferase, calcium, inorganic phosphorus, sodium, potassium, chloride, total protein, albumin, globulin & albumin/globulin ratio) • Urinalysis (specific gravity, volume, color, appearance, pH, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, erythrocytes, leukocytes & sediment) • Full autopsy on all animals: macroscopic examination, organ weight & histological examination (adrenal glands, aorta, bone marrow, brain, epididymides,	(Canut et al., 2012)

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	esophagus, eyes with optic nerve, femur, heart, intestines, kidneys, larynx, liver, lungs, lymph nodes, mammary gland area, ovaries, pancreas, pituitary gland, prostate and seminal vesicles, salivary glands, sciatic nerve, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid, trachea, urinary bladder, uterus, vagina & all gross lesions) **Results and Significance:** • No significant adverse effects were observed in food consumption, body weight, mortality, clinical biochemistry, hematology, gross pathology or histopathology. • Minor differences were observed between the treated and control animals in body-weight gain (%) and some hematological blood parameters but were not dose-related or of toxicological significance. • Relative liver (to body) weight was significantly higher in low and high-dose females compared to controls, although this effect was not observed in male animals. Thus, the authors considered the increases in relative liver to body weights to be of no biological significance due to the lack of effects in males, the absence of histopathological correlates, and the lack of effects on biochemical markers of liver function. • NOAEL=600 mg RCE/kg bw/day (360 mg NaHA/kg bw/day)	
Study Design: 90-d subchronic oral toxicity **Study Length:** 90 days **Animals:** 80 Sprague-Dawley rats (10M/10F per group) **Dose/Delivery/Vehicle/Frequency:** 1x daily by gavage at 0,30, 300 or 1000 mg/kg bw chicken sternal cartilage preparation (BioCell Collagen II®; 10% HA; MW=NS) suspended in distilled H_2O	**Outcome Measurements:** • Mortality, body weight & food intake • Hematological testing (hematocrit, hemoglobin concentration, erythrocyte count, total white blood cell count, differential leukocyte count, platelet count, absolute reticulocyte count, mean corpuscular hemoglobin, red cell distribution width, mean corpuscular volume, prothrombin time & activated partial thromboplastin time) & blood biochemistry (albumin, alkaline phosphatase, blood creatinine, calcium, chloride, globulin, glucose, inorganic phosphorus, potassium, alanine aminotransferase, aspartate aminotransferase, sodium, sorbitol dehydrogenase, total bilirubin, total cholesterol, total protein, triglycerides & blood urea nitrogen) • Full autopsy on all animals: macroscopic examination (external surfaces and orifices, and cranial, thoracic, and abdominal cavities and their contents), organ weight (liver, kidneys, adrenals, brain, heart, thymus, spleen, uterus, ovaries, testes & epididymides) &	(Schauss et al., 2007)

STUDY SETUP AND DETAILS	ANIMAL STUDY DETAILS AND RESULTS	REFERENCE
	histological examination [gross lesions, lungs, trachea, brain (including sections of the medulla/pons, cerebellar cortex and cerebral cortex), spinal cord (three levels: cervical, mid-thoracic, and lumbar), eyes, pituitary, thyroid/parathyroid, thymus, heart, aorta, sternum with bone marrow, liver, spleen, kidneys, pancreas, adrenals, ovaries, testes, uterus, vagina, accessory genital organs (epididymides, prostate, and seminal vesicles), female mammary gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, representative lymph node (mesenteric and mandibular), salivary glands, peripheral nerve (sciatic) & skin] **Results and Significance:** • No deaths • Normal growth & feeding • No dose-related or toxicologically significant changes in any of the treated animals • No significant effects on hematological data at any dose • Small changes in the biochemical analyses considered to be unrelated to treatment due to the absence of a dose relationship • Two significant changes in organ weights: a slightly higher brain weight in females in the low dose group and a lower brain to spleen weight ratio in males in the intermediate dose group. Considered unrelated to treatment due to no correlating or other significant changes in organ weights in the high dose group. • No gross or microscopic abnormalities were found after terminal sacrifice • NOAEL=1000 mg/kg bw/day (chicken sternal cartilage preparation) or (100 mg NaHA/kg bw/day)	

ALT – alanine aminotransferase, BUN – blood urea nitrogen, LD_{50} – median lethal dose, MTD – maximum-tolerated dose, MW – molecular weight, NOAEL – no-observed-adverse-effect-level, NS – not specified, RCE – rooster combs extract, TG – triglycerides

5. Human Studies with NaHA and HA

Two published (Tashiro et al., 2012; Bellar et al., 2019) human studies that evaluated the oral intake of NaHA (MW 35-900 kDa) produced by bacterial fermentation were identified. One additional unpublished human study was conducted for Bloomage (Hunan Provincial Center for Disease Control and Prevention, 2012). Four additional studies evaluating the oral intake of RCE containing 10% (Nagaoka et al., 2010), 60-70% (Kalman et al., 2008) or 60-80% (Martinez-Puig et al., 2013; Sola et al., 2015) HA or NaHA were also considered. For the purpose of evaluating the safety and tolerability of NaHA, the focus is on any potential adverse effects associated with their intake. Studies reviewed are presented in Table 10.

Hyaluronic acid

A randomized, double-blind, placebo-controlled clinical trial was conducted on the efficacy of oral HA administration for osteoarthritis in knee joints (Tashiro et al., 2012). Sixty osteoarthritic subjects were assigned either 200 mg/d HA in capsule form (Kewpie Corporation (Tokyo); purity=97%; MW=900 kDa) or placebo (cornstarch) for 12 months. No adverse effects were reported by the authors.

In a prospective longitudinal trial[8], the safety and tolerability of oral NaHA (~35 kDa; produced by microbial fermentation) was assessed in healthy human subjects (Bellar et al., 2019). Twenty young adults (9M/11F; 30.7±5.6 y) were administered 140 mg pharmaceutical grade HA35 once daily for 7 days (Bellar et al., 2019). Subjects were then followed for a total of 28 days. HA35 was well tolerated by all subjects with no serious adverse events reported over the course of the trial, although several subjects experienced mild to moderate bloating (N=3) and cramping (N=8) that resolved several hours after dosing. There was no placebo group in this trial, however, to allow for comparison. Quigley et al. compared the prevalence of abdominal cramping and pain in nine countries (Quigley et al., 2006). The frequency of episodes differed between countries being the highest in the US (61% suffered at least once per week). The 40% of subjects that reported cramping in the Bellar et al. trial, therefore, is within the normal US range reported by Quigley et al. No statistically significant changes were observed compared to baseline in clinical (BP, HR, blood glucose, serum amino transferases, blood urea nitrogen, serum creatinine, albumin, bilirubin), biochemical (serum hsCRP, TNF$\propto$, IL6; fecal calprotectin & β-defensin 2; serum LPS) or metabolic function tests or in microbiome composition.

An unpublished oral human safety study of NaHA-containing capsules was conducted by the Hunan Center for Disease Control and Prevention for Bloomage (Hunan Provincial Center for Disease Control and Prevention, 2012). The molecular weight of the NaHA used in this study was 330 kDa and the capsule contained a mixture of NaHA and an unspecified oligopeptide. In this 45-day study, 52 female subjects received placebo and 52 received capsules containing a mixture of NaHA, oligopeptide and procyanidine to yield a daily dose equivalent to 100 mg/d NaHA. No abnormal changes in body weight, blood pressure or heart rate were associated with NaHA intake. In addition, there were no abnormalities in blood (RBC, WBC, hemoglobin), urine (pH, white cell, urine sugar) or stool tests. Biochemical tests (albumin, ALT, BUN, cholesterol, triglyceride, creatinine, uric acid, glucose, aspartate aminotransferase), electrocardiograms, abdominal ultrasound examinations and chest x-rays were all within normal ranges. No allergy or any other adverse reactions were observed. The results of this study suggest that 100 mg/d NaHA is well-tolerated.

Rooster comb or cartilage extracts

The effect of dietary NaHA on pain relief and quality of life in patients with knee osteoarthritis was evaluated using Hyal-Joint®, an encapsulated dietary ingredient extracted from chicken combs containing 60-70% NaHA (purity and molecular weight not stated) (Kalman et al., 2008). Twenty subjects (≥40 y) with knee osteoarthritis received either 80 mg/d Hyal-Joint® (~48 mg NaHA/d) or placebo for 8 weeks. Tolerability and safety parameters included the incidence and severity of adverse events and changes in blood pressure and heart rate, as well as blood cell count and biochemical profile (not specified). No significant changes were observed in vital signs, body weight, blood cell count or biochemical profile in the patients who received Hyal-Joint®.

[8] ClinicalTrials.gov, Human Pilot Study – HA35 Dietary Supplement for Promoting Intestinal Health, NCT02867605.

Human studies have also been performed on Bioiberica's RCE (IB0004) (Martinez-Puig et al., 2013; Sola et al., 2015). In a prospective, randomized, double blind placebo-controlled nutrition intervention trial, the safety of the oral administration of yogurt supplemented with RCE (Mobilee™) in healthy adults was investigated. Bioiberica's RCE contains 60-80% NaHA with a molecular weight of approximately 800 kDa. In a preliminary study, two groups of 20 adults received either unsupplemented yogurt or yogurt supplemented with 80 mg of RCE every day (~48 mg NaHA/d) for 90 days (Martinez-Puig et al., 2013). During the study period, no adverse events were reported, and no changes were observed in body weight, blood pressure or pulse rate in either group. Isokinetic testing showed that the RCE group had improved joint mechanics and muscle function. In a follow-up study, two groups of 40 adults received either unsupplemented yogurt or yogurt supplemented with 80 mg of RCE every day (~48 mg NaHA/d) for 12 weeks (Sola et al., 2015). Adverse events were reported in 9 subjects and were related to GI discomfort such as flatulence and stomachache. The severity of the adverse events was mild and in none of the cases was the intervention modified or interrupted. No statistically significant differences were observed between the control and intervention groups with respect to adverse events reported.

The effects of a RCE (~10% HA) on symptoms of knee osteoarthritis and cartilage metabolism were examined in a randomized, double-blind, placebo-controlled, parallel study conducted in subjects with osteoarthritis of the knee (Nagaoka et al., 2010). Forty-three study subjects (8M, 35F; 40-85 y) were randomized to receive placebo or capsules containing a total of 630 mg/d RCE (~60 mg HA) for 16 weeks. Safety and tolerability were assessed by adverse event reporting, hematological examination, blood biochemistry profile, urinalysis, blood pressure changes and pulse rate. No serious adverse events were reported, and no adverse effects were attributed to the RCE. No changes in body weight, vital signs or laboratory parameters were observed compared to baseline.

Table 10. Summary of Human Studies for NaHA and HA

STUDY SETUP AND DETAILS	HUMAN STUDY RESULTS, SIGNIFICANCE, SAFETY	REFERENCE
Study Design: Randomized, double-blind, placebo-controlled clinical trial **Study Length:** 12 months Subjects: 60 osteoarthritic subjects (30 placebo/30 HA)with knee joint pain aged >50 y **Dose/Delivery/Frequency:** 4x 50mg HA capsules for a daily dose of 200 mg/d HA (97% purity; MW=900 kDa); placebo=cornstarch	**Outcome Measurements:** • Osteoarthritis symptoms evaluated by the Japanese Knee Osteoarthritis Measure (JKOM) score **Results and Significance:** • No adverse effect of treatment on outcomes measured **Safety Measurements/Adverse Events Reported:** • No adverse effects reported	(Tashiro et al., 2012)
Study Design: Prospective, longitudinal trial[1] **Study Length:** 7 days with 28 d follow-up **Subjects:** 20 (9M/11F; 30.7±5.6 y) **Dose/Delivery/Frequency:** 1x daily 140 mg pharmaceutical grade HA35 (fermentation; MW=35 kDa) dissolved in sterile H_2O	**Outcome Measurements:** • BP & HR • Clinical laboratory tests: blood glucose, serum aminotransferases, BUN, serum creatinine, albumin & bilirubin • Stool microbiome diversity • Serum cytokines: hsCRP, TNFα & IL-6 • Intestinal permeability: serum LPS	(Bellar et al., 2019)

STUDY SETUP AND DETAILS	HUMAN STUDY RESULTS, SIGNIFICANCE, SAFETY	REFERENCE
	• Antimicrobial stool peptide: calprotectin **Results and Significance:** • No statistically significant changes were observed compared to baseline in clinical, biochemical or metabolic function tests or in microbiome composition **Safety Measurements/Adverse Events Reported:** • HA35 was well tolerated by all subjects; no serious adverse events, although several subjects experienced mild to moderate bloating (N=3) and cramping (N=8) that resolved several hours after dosing.	
Study Design: Randomized, double-blind, placebo-controlled trial **Study Length:** 45 days **Subjects:** 104 females aged 30-50 y (52 placebo/52 NaHA) **Dose/Delivery/Frequency:** 2x daily of 3 capsules containing mixture of NaHA (MW=330 kDa), unspecified oligopeptide & procyanidine; daily dose equivalent of 100 mg/d NaHA	**Outcome Measurements:** • Physical exam: weight, blood pressure, heart rate • Hematologic parameters: RBC, WBC, hemoglobin • Urinalysis: pH, white cell, urine sugar • Stool examination: Fecal egg counts • Biochemical tests: glucose, triglyceride, cholesterol, BUN, creatinine, uric acid, alanine aminotransferase, aspartate aminotransferase, total protein, albumin • Electrocardiogram, abdominal ultrasound & chest x-ray **Results and Significance:** • No significant differences in weight, blood pressure or heart rate between placebo and test groups • After 45 days, all urinalysis, stool exam, hematologic & biochemical assay results were within the normal range • No abnormalities were observed in electrocardiogram, abdominal ultrasound or chest x-ray • Suggests that 100 mg/d NaHA is well-tolerated **Safety Measurements/Adverse Events Reported:** • No obvious allergic or other adverse reaction was observed during the trial	(Hunan Provincial Center for Disease Control and Prevention, 2012)
Study Design: Randomized, double-blind, placebo-controlled single center study **Study Length:** 8 wk **Subjects:** 20 subjects ≥40 y (10 placebo/10 active product) **Dose/Delivery/Frequency:** 1x daily capsule of 80 mg Hyal-Joint®, a dietary ingredient extracted from chicken combs containing 60-70% NaHA (purity & MW = NS); equivalent to 48 mg NaHA/d	**Outcome Measurements:** • Changes in BP & HR • Blood cell count & biochemical profile (NS) • Incidence & severity of adverse events **Results and Significance:** • No significant changes in vital signs, body weight, blood cell count or biochemical profile **Safety Measurements/Adverse Events Reported:** • One adverse event was reported during study period in the active product group. One subject complained of acute non-target knee pain, unrelated to the study product, and voluntarily dropped out of the study.	(Kalman et al., 2008)
Study Design: Prospective, randomized, double-blind, placebo-controlled study	**Outcome Measurements:** • Body weight, BP & pulse rate • Incidence & severity of adverse events	(Martinez-Puig et al., 2013)

STUDY SETUP AND DETAILS	HUMAN STUDY RESULTS, SIGNIFICANCE, SAFETY	REFERENCE
Study Length: 90 d **Subjects:** 40 healthy adults with joint discomfort (20 placebo/20 RCE-supplemented) **Dose/Delivery/Frequency:** 1x daily yogurt supplemented with 80 mg RCE (Bioiberica; Mobilee™; 60-80% NaHA; MW=800 kDa) or placebo of unsupplemented yogurt; equivalent to 48 mg NaHA/d	**Results and Significance:** • No changes observed in body weight, BP or pulse rate • Isokinetic testing showed improved joint mechanics and muscle function **Safety Measurements/Adverse Events Reported:** • No adverse events were reported	
Study Design: Randomized, double-blind, placebo-controlled, parallel study **Study Length:** 12 wk **Subjects:** 80 healthy adults with mild knee pain (30M/50F) and mean age of 42.5±13.2 y **Dose/Delivery/Frequency:** 1x daily yogurt supplemented with 80 mg RCE (Bioiberica; Mobilee™; 60-80% NaHA; MW=800 kDa) or placebo of unsupplemented yogurt; equivalent to 48 mg NaHA/d	**Safety Measurements/Adverse Events Reported:** • Adverse events were reported in 9 subjects and were related to GI discomfort such as flatulence and stomachache. The severity of the adverse events was mild and in none of the cases was the intervention modified or interrupted. No statistically significant differences were observed between the control and intervention groups with respect to adverse events reported.	(Sola et al., 2015)
Study Design: Randomized, double-blind, placebo-controlled, parallel study **Study Length:** 16 wk **Subjects:** 43 subjects with osteoarthritis of the knee (8M/35 F; 40-85 y) **Dose/Delivery/Frequency:** 6x daily 105 mg RCE-containing capsules for a daily dose of 630 mg RCE (~60 mg HA; MW=NS) or vehicle-only placebo (crystalline cellulose, dextrin & fatty acid sugar esters)	**Outcome Measurements:** • Hematological examination • Blood biochemistry profile • Urinalysis • Blood pressure changes & pulse rate • Incidence & severity of adverse events **Results and Significance:** • No changes in body weight, vital signs or laboratory parameters with RCE compared to baseline **Safety Measurements/Adverse Events Reported:** • No serious adverse events were reported & no adverse effects were attributed to the RCE	(Nagaoka et al., 2010)

[1] ClinicalTrials.gov, Human Pilot Study – HA35 Dietary Supplement for Promoting Intestinal Health, NCT02867605.
BP – blood pressure, BUN – blood urea nitrogen, HR – heart rate, hsCRP – high-sensitivity C-reactive protein, IL-6 – Interleukin-6, JKOM – Japanese Knee Osteoarthritis Measure, NS – not specified, RCE – rooster combs extract, RBC – red blood cells, TNF-α – tumor necrosis factor-α, WBC – white blood cells

6. Reproductive/Developmental Effects of NaHA and HA

Reproductive and development studies are important for GRAS assessments because the concept of GRAS presumes safety for the general population. Effects on potentially sensitive populations such as pregnant women cannot be excluded.

A comprehensive review of the reproductive toxicity of NaHA, concluded that hyaluronic acid is a naturally occurring glycosaminoglycan and is not expected to adversely affect pregnancy outcome (REPROTOX, 2018). This conclusion was based on no adverse outcomes in rat and rabbit fertility and pregnancy studies at doses ranging from 40-64 mg/kg/day (Ono et al., 1992; Ota et al., 1991; Tanaka et al., 1991a; Tanaka et al., 1991b; Tateda et al., 1992; Wada et al., 1991; Kumada et al., 1995; Matsuura et al., 1994c; Matsuura et al., 1994b; Matsuura et al., 1994a; Hattori et al., 1995); the normal presence of HA in female reproductive tract fluids as well as being a constituent of the cumulus matrix, zona pellucida and perivitelline space; and the role that HA appears to play in oocyte maturation and cumulus expansion (Fenton et al., 1993; Chen et al., 1993; Chen et al., 1990) and its ability to promote sperm function including motility, capacitation and acrosome reaction (Kawakami et al., 2000).

Oral sodium hyaluronate

Bloomage sponsored teratogenicity testing of NaHA (MW 270kDa) in rats (unpublished) by the Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention (Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007a). Sixty female Wistar rats were administered intragastric doses of 0, 170, 330 or 670 mg/kg bw/day, respectively, for 10 days (during days 7-16 of gestation). The pregnant dams grew normally and were euthanized on day 20 of gestation. The uterus and ovary were weighed and the numbers of corpus lutea, nidation, living embryos, dead embryos & absorbed embryos were recorded. The fetal rats and placentas were weighed and measured and the fetal rats were examined for the systematic and developmental condition of the head, spine, chest, abdomen, tail and limbs. Visceral examinations of the fixed fetal rats included the brain, jaw, trachea, esophagus, spinal cord, heart, lung, diaphragm, liver, stomach, intestine, kidney, bladder and uterus or testis. Skeletal development examinations included the occipital bone, vertebra, pelvis, limb bones, carpal bones, metacarpal bones, bones of toes, costal bones, and sternum. No significant differences ($P>0.05$) were observed in the maternal, uterus and ovary weights or in the number of corpus lutea and nidation between the test and control groups. No significant differences ($P> 0.05$) were detected between the test groups and the control group on the weight and length of living embryos, the weight of the placenta, and the number of living embryos, dead embryos and absorbed embryos in a brood. The number of fetal rats with normal skeleton development in the test groups was higher than that in the control group. No abnormality of the visceral organs due to NaHA was detected. The authors concluded, therefore, that no evidence of maternal toxicity, embryo toxicity or effects on fetal skeletal development resulted from NaHA administration.

A teratogenicity study of NaHA was conducted in SD rats (Peng et al., 2011). Pregnant rats were divided into five groups (N>12); three groups received 333, 667 or 1,333 mg/kg bw NaHA, one group received water as a negative control, and one received 250 mg/kg bw aspirin as a positive control. Animals were dosed by oral gavage once per day during gestation days 7-16. On day 20 of gestation, the pregnant rats were sacrificed, and embryonic and fetal development were evaluated. No significant differences in maternal body weight or total weight were observed in NaHA treated rats compared to the negative controls. NaHA did not affect either the number of corpus lutea or

implantations of the test groups. No statistical differences in uterus weight, placental weight, live fetus rate, fetal death rate or absorbed fetus rate were found. Fetal development and growth (*i.e.*, body weight, body length, tail length and skeletal development) in the test groups were not statistically different than in the negative controls. No abnormalities were observed in the entrails, head, trunk, tail, arms and legs of the fetal rats. The authors concluded that NaHA caused neither maternal toxicity nor teratogenicity at doses up to 1,333 mg/kg bw/d during gestation days 7-16.

Subcutaneous hyaluronic acid

Several reproductive and developmental studies of subcutaneous NaHA (SL-1010) produced by bacterial fermentation were identified. At doses of 50 mg/kg bw/day by subcutaneous administration, HA has not been shown to negatively affect the fertility and pregnancy outcomes in rats and rabbits (Tanaka et al., 1991a; Tanaka et al., 1991b; Ota et al., 1991; Wada et al., 1991). *In vitro* studies show that hyaluronic acid aids in the development of embryos in cows and mice (Hattori et al., 1995; Furnus et al., 1998). Although subcutaneous studies may have limitations when evaluating oral administration, NaHA has low oral availability (~10%). It would be expected that subcutaneous administration would provide fetal exposure at a higher dose than possible via oral administration. As a result, a lack of reproductive and teratogenic effects following subcutaneous administration supports the safety of oral administration.

Several reproductive and developmental studies in Crj:SD rats were conducted to determine the effects of SL-1010 on males, females and their fetuses. The first study was a fertility study. SL-1010 was administered subcutaneously at doses of 0, 5, 15 or 50 mg/kg bw/day to male rats 60 days before mating and then throughout the mating period (Tanaka et al., 1991a). The same doses were administered to female rats from 14 days before mating until day 7 of pregnancy. All pregnant rats were sacrificed on day 20 of pregnancy to examine fetal development. There were no gross findings at necropsy except for swellings at injection sites. Both males and females receiving the highest dose showed increases in body weight though the test period, an observation that was attributed to retention of the test solution in subcutaneous tissue. SL-1010 did not affect copulation or fertility, and no abnormalities in the fetuses externally, skeletally or viscerally were detected. From this study, the NOEL of SL-1010 was determined to be 50 mg/kg bw, the highest dose tested.

A second teratogenicity study was conducted in Crj:SD rats to determine the effects of SL-1010 on dams and their first generation offspring (Tanaka et al., 1991b). Subcutaneous injections of SL-1010 at doses of 5, 15 or 50 mg/kg bw/day were given to females from days 7 to 17 of pregnancy. A group of dams from each dose level was sacrificed on day 20 of pregnancy to examine the development of fetuses. Furthermore, a group of dams from each dose carried to term was sacrificed to examine postnatal growth and development of their offspring. No changes in the general condition of the rats were observed with the exception of injection site swellings. There were no effects on the duration of pregnancy, delivery or nursing conditions at any dose level. There were no abnormal gross findings at caesarean section and no abnormal growth of fetuses. In the examination of live births, no skeletal or visceral abnormalities were detected, nor were there abnormalities in body weight, growth, learning ability or reproductive ability in any of the treated groups. From this study, the NOEL of SL-1010 for pregnant rats and their first-generation progeny was determined to be 50 mg/kg bw, the highest dose tested.

A perinatal and postnatal study in Crj:SD rats was conducted to determine the effects of SL-1010 on female rats and their offspring (Ota et al., 1991). Four groups of pregnant rats received subcutaneous doses of SL-1010 of 0, 5, 15 or 50 mg/kg bw/day from day 17 of pregnancy to day 21

after delivery. The dams receiving the highest dose had an increased weight gain during the dose period that was attributed to the retention of the test solution in the subcutaneous tissue. There were no changes in general condition, food consumption, parturition or lactation of the dams in any group. In the offspring, there were no changes in viability, growth, behavior and functional development, sexual maturation or reproductive ability. In addition, no changes were found in the development of F_2 generation pups. From this study, the NOEL for SL-1010 for maternal rats and their offspring was determined to be 50 mg/kg bw, the highest dose tested.

A teratogenicity study was conducted in rabbits to determine the effects of SL-1010 on dams and their fetuses (Wada et al., 1991). Four groups of 13 to14 pregnant rabbits subcutaneously received 0, 5, 15 or 50 mg/kg bw/day from day 6-18 of pregnancy. There were no changes in the general condition of the pregnant rabbits throughout the study. Dams in the 15 and 50 mg/kg bw group showed an increase in body weight gain during the dose period; this was attributed to a retention of test solution at the injection site. There were no abnormal changes in the mortality or growth of fetuses in any treated group, nor were teratogenic effects of SL-1010 observed in any group. The NOEL for SL-1010 for both pregnant rabbits and their fetuses was established to be 50 mg/kg, the highest dose tested.

7. Carcinogenicity of NaHA and HA

HA is responsible for various functions within the extracellular matrix such as cell growth, differentiation and migration (Necas et al., 2008). Two of the major HA-binding receptors include a cell-surface glycoprotein, CD44, that has diverse roles in cell-cell and cell-matrix interactions and the receptor for HA-mediated motility (RHAMM) that is found intracellularly in the cytoplasm, in the nucleus, and on the cell surface. Under normal physiological conditions, the amount of HA is controlled by a balance between synthesis and degradation. However, elevated levels of HA often occur in the surrounding stroma of malignant tumors (Sironen et al., 2011). The HA level is also elevated in various cancer cell lines (Lin and Stern, 2001). This HA-rich microenvironment may promote tumor progression by enhancing cell proliferation, migration, invasion, metastasis, angiogenesis and resistance to chemotherapeutic agents (Hill et al., 2006; Sato et al., 2016). Clinically, HA expression in tumor cells is associated with poor survival in patients with gastric and colorectal cancers and high levels of stromal HA are associated with poor survival rates in patients with prostate, breast and ovarian cancers (Sato et al., 2016).

Despite the apparent role of endogenous HA dysregulation in cancer progression, little is known about the carcinogenicity of increased systemic levels of HA resulting from exogenously administered HA (*i.e.*, oral or subcutaneous). No studies evaluating the carcinogenicity of oral NaHA or RCE were identified. However, the causal role of HA in tumor formation, progression, and metastasis was reviewed and discussed by the Cosmetic Ingredient Review (CIR) Expert Panel (Becker et al., 2009).

Conflicting evidence has been published regarding tumor growth in HA-injected mice. The subcutaneous administration of 0.5 μg/0.05 μL/h HA (MW 80-2000 kDa) to Balb/c nu/nu male mice or A/Jax mice for 7 to 14 days resulted in the inhibition of LX-1 tumor growth by 50 to 80% from LX-1 tumor cells and TA3/St tumor growth by 60 to 65% from TA3/St tumor cells that had been injected in front of the HA site (Ghatak et al., 2002). In a separate study, the intraperitoneal injection of 30 mg/kg HA (MW not provided) had no effect on the lifespan of female mice inoculated intraperitoneally with U14 cervical tumor cells when compared with control mice injected with saline (Yin et al., 2006). Yin et al. (2006) also reported that tumor metastasis was reduced in female C57BL/6 mice implanted

with Lewis cell carcinoma cells in the footpad that had been treated with 30 mg/kg HA intraperitoneally for 5 days compared to those treated with saline. In contrast to the results of Ghatak et al. (2002) and Yin et al. (2006), larger tumors and increased tumor weights were reported in mice injected in the back with 0.2 ml of 5% HA (MW not provided) and adenocarcinoma cell line colon 26 in 0.1 ml HA compared to the control mice injected with 0.2 ml saline and adenocarcinoma cell line colon 26 in phosphate buffered saline (Matsui et al., 2004). The expression of CD44 on the surface of the cancer cells was also increased in the HA group compared to the control group.

Stabilin-2 (Stab-2) is a receptor that binds and clears HA from the circulation. A Stab-2 knock-out (KO) mouse has significantly higher serum HA levels than wild-type mice (Hirose et al., 2012). The metastasis of B16F10 melanoma cells administered intravenously was significantly reduced in Stab2 KO mice compared to wild-type mice. To confirm this finding, wild-type mice were administered a monoclonal antibody (mAb) to Stab2 that significantly increased serum HA levels. Metastasis of B16F10 cells injected intravenously was significantly inhibited in mice administered the anti-Stab2 mAb compared to wild-type mice administered IgG. The authors concluded that serum HA levels are inversely correlated with tumor metastasis.

After considering a large amount of data, the results of two studies were considered pivotal by the CIR Expert Panel in forming their conclusion regarding the role of HA in cancer metastasis (Becker et al., 2009). In the first study it was reported that a reduced level of HA was associated with an unfavorable prognosis of clinical stage 1 cutaneous melanoma (Auvinen et al., 2000). In the second study, the expression of CD44 and HA levels were examined in paraffin-embedded samples of basal cell carcinomas, *in situ* carcinomas, squamous cell carcinomas and normal epidermis (Karvinen et al., 2003). In basal cell carcinomas, CD44 expression was quite low. In squamous cell carcinomas, CD44 expression was variable. As the malignancy became less differentiated and, thus, be expected to have a higher risk for metastasis, the expression of HA decreased. The CIR Expert Panel cited these key findings in their conclusion "that HA likely does not play a causal role in metastasis and that increased expression of HA genes may be a consequence of metastatic growth not the converse."

8. Toxicology of Glucosamine

N-acetylglucosamine is a metabolite of NaHA. Therefore, the toxicology of glucosamine should be considered as part of the safety assessment of NaHA. A comprehensive scientific review by Anderson et al. (2005) summarized the extensive research on glucosamine in animals and humans (Anderson et al., 2005).

Glucosamine is a component of all mammalian connective tissue that is synthesized from glucose and forms mucopolysaccharides. Because of its high concentration in joint tissues, glucosamine supplements are widely used to relieve the symptoms of osteoarthritis. Three forms of glucosamine that are derived from marine invertebrate chitin are commonly available as supplements: glucosamine hydrochloride, glucosamine sulfate, and N-acetylglucosamine. Oral glucosamine is 90% absorbed and a significant fraction of orally administered glucosamine undergoes first-pass metabolism in the liver. Glucosamine is a prominent component of hexosamine pathway, an important branch of glycolysis. Exogenous glucosamine is actively transported from extracellular tissue into cells by glucose transporters. Insulin facilitates glucosamine transport into cells where it is phosphorylated by one of a family of hexokinases to glucosamine-6-phosphate (Anderson et al., 2005).

Acute oral administration of glucosamine in laboratory animals at large doses (5,000–15,000 mg/kg bw/day) is well-tolerated without documented toxicity. The oral LD_{50} for glucosamine in rats and mice

was 5,000 mg/kg bw/day in all studies. In oral subchronic and chronic toxicity studies, glucosamine was well-tolerated in rats, mice, rabbits and dogs at doses of approximately 159-8,000 mg/kg bw/day for 12-365 days. The NOAEL for rats was determined to be 2700 mg/kg bw/day (365 days) and is 2149 mg/kg bw/day in dogs (183 days). In addition, oral administration of large doses of glucosamine in animals has no documented effect on glucose metabolism (Anderson et al., 2005).

Anderson et al. (2005) also reviewed the results from 32 clinical trials to examine the potential toxicity of glucosamine to humans (Anderson et al., 2005). These studies included 3063 subjects for an average of 17 weeks. Sixteen of these studies demonstrated that glucosamine administration for up to 37 weeks did not adversely affect glucose metabolism. In other studies, healthy subjects exhibited no adverse effects from an infusion of 9.7 g, and only one subject developed a headache after an infusion of 30.5 g. In 13 clinical trials reporting safety parameters, there were no adverse effects of glucosamine on blood chemistry, hematologic markers, urinalysis, occult blood in feces or cardiovascular parameters. Thus, in numerous randomized, double-blind, placebo-controlled human studies with doses up to 2,000 mg/day, conducted in both normal subjects and in individuals suffering from type 2 diabetes, no evidence of glucosamine toxicity, intolerance or adverse effects on glycemic control was noted. Adverse effects reported by individuals receiving glucosamine have not differed in incidence, character or severity from those experienced by controls receiving placebos. The authors concluded that glucosamine is safe under current conditions of use as a dietary supplement based on numerous animal toxicology studies and human clinical studies.

9. Allergenicity Potential of Sodium Hyaluronate

In the 1960s, it was acknowledged that animal-derived HA sources can contain proteins or other impurities that may cause allergic inflammatory responses. Bacterial HA is an alternative source. The HA polymer produced by animals and bacteria is identical, except that bacterial HA is not immunogenic. Bacteria known to synthesize HA are mostly from *Streptococci* species that are able to digest blood-based agar medium to produce a slimy translucent layer via HA synthesis that surrounds the bacterial colonies. This HA “capsule” likely prevents the bacteria from being recognized as a foreign entity by a host immune system. The extraction of HA from microbial fermentation broth is a relatively simple process that results in high yields. Therefore, HA production using pathogenic *Streptococci* or a safe recombinant microbe is now preferred over extraction from animal tissue (Schiraldi et al., 2010).

No reports of allergy from oral consumption of NaHA or HA were found in the literature. As a carbohydrate, NaHA is not expected to exhibit any allergenic activity. A review article summarized this point by stating, “NaHA and HA are highly non-antigenic and non-immunogenic, owing to its high structural homology across species, and its poor interaction with blood components (Necas et al., 2008).”

10. Safety of *Streptococcus equi* subsp. *zooepidemicus*

Streptococcus zooepidemicus is a subspecies of *Streptococcus equi* and is a normal bacteria flora in horses (Lindmark et al., 2001). This microorganism has also been isolated from wound infections of horses and from other mammals such as cows and swine (Gaviria and Bisno, 2000). Infections in humans from *Streptococcus equi* subsp. *zooepidemicus* are reported to be rare but have occurred. Several reports indicate that the most common vehicle for infections in humans is unpasteurized milk (Barnham et al., 1983; Edwards et al., 1988). Infectious strains have been characterized (Barnham et al., 1987a; Barnham et al., 1987b).

Traditionally, HA for use in pharmaceuticals and foods was extracted from rooster combs, but now it is mainly produced via streptococcal fermentation due to lower production costs and less environmental pollution (Liu et al., 2011). The production of HA by Streptococcus was first demonstrated by Kendall et al. (Kendall et al., 1937) and microbial HA production on an industrial scale was first achieved in the 1980s by Shiseido (Liu et al., 2011). *Streptococcus equi* subsp. *zooepidemicus* remains the most common strain for industrial production of HA (Chong and Nielsen, 2003; Krahulec and Krahulcova, 2006). However, recombinant HA production in other strains, including *Bacillus sp.*, *Lactococcos lactis*, *Agrobacterium sp.*, and *Escherichia coli*, is emerging as an alternative approach (Liu et al., 2011). The presence of bacterial endotoxins in HA from streptococcal fermentation limits the application of HA in parenteral preparations. The European Pharmacopoeia limit for bacterial endotoxins in NaHA is "less than 0.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins and less than 0.05 IU/mg, if intended for use in the manufacture of intra-ocular preparations or intra-articular preparations without a further appropriate procedure for the removal of bacterial endotoxins (European Pharmacopoeia, 2010)." After oral administration, endotoxin is degraded and metabolized into small molecules in the digestive system. No endotoxin limit for NaHA produced by bacterial fermentation is specified in the current FCC guidelines (Food Chemicals Codex, 2018).

Streptococcus equi subsp. *zooepidemicus* still remains the most common strain used for industrial HA production and has been accepted as a fermentation organism for biomedical use in Europe (European Pharmacopoeia, 2010) and Korea (Korean Ministry of Food and Drug Safety, 2014) and in FCC specifications (Food Chemicals Codex, 2018). All industrial processes inactivate the organism and remove bacterial fragments based on several common steps.

Streptococcus equi subsp. *zooepidemicus* is inactivated and removed through the purification and refining processes outlined in Figure 2. Bloomage states that *Streptococcus equi* subsp. *zooepidemicus* cannot survive under conditions of high temperature (*i.e.*, >45°C (Vos et al., 2009)) and ethanol concentration (> 50%). In the process used by Bloomage, the organism is inactivated by 50-80% ethanol precipitation (see experimental report in Appendix 7). The crude NaHA is then dissolved in water and filtered in a plate and frame filter press. A clear filtrate is obtained after the residual production organism is removed. Yearly, Bloomage tests multiple batches of NaHA for hemolytic streptococcus and other strains, including *S. aureus*, *Salmonella*, etc. A sample yearly test report is included in Appendix 6. Every batch is tested for bacteria counts, mold and yeast, *E. coli*, *Staphylococcus aureus* and *Salmonella*.

C. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "…reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use."[9]

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is

[9] See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 4/15/17).

FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "…General recognition of safety requires common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food that there is reasonable certainty that the substance is not harmful under the conditions of its intended use."
>
> "'Common knowledge' can be based on either 'scientific procedures' or on experience based on common use in food prior to January 1, 1958." [10]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[11]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Renwick, 1990; Rulis and Levitt, 2009; Lu, 1988).

As noted below, this safety assessment to ascertain GRAS status for Bloomage's NaHA for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

D. FDA Safety Methodology

Safety assessment methodology has been defined by advances in the science of risk assessment. Risk assessment, simply defined, consists of an estimate of exposure to a chemical or food ingredient coupled with an assessment of assigning a safe dose or level of exposure. Exposure estimates are

[10] See 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 4/15/17).
[11] See Footnote 3.

based on knowledge of how the chemical and ingredient will be used. Assigning a safe dose can be a highly scientific mathematical approach, or a judgment approach, or a blend of these two approaches. The approach is usually dictated by the quantity, quality and rigor of the safety data available. For example, assessment of carcinogenic risk is usually a highly mathematical approach relying on specialized safety data. GRAS assessments on history of use are more a function of judgment based on information about use, as opposed to analysis of safety data. For ingredients where there is no history of use, FDA has traditionally used an approach that relies on simple mathematics using safety data and some measure of scientific judgment (Kokoski et al., 1990). FDA primarily relies on the review of laboratory animal data. More recently, FDA is relying on human clinical information. FDA toxicologists first determine that the study does not demonstrate any indication of a carcinogenic effect. The next step is to carefully review the findings at each dose level and assign the dose level without adverse effects as the NOAEL or "no adverse effect level." The NOAEL, expressed as a weight of ingredient per kilogram of body weight of the experimental animal, is divided by an appropriate safety factor to obtain an acceptable daily intake (ADI). The ADI is then compared to an estimated daily intake (EDI), expressed in the same units for sake of comparison. If the ADI comfortably exceeds the EDI, the ingredient is considered to be safe under intended conditions of use. If the ADI and EDI are close to being equivalent, or even if the EDI slightly exceeds the ADI, scientific judgment based on a variety of factors can be used to consider the ingredient to be safe under intended conditions of use (Frankos and Rodricks, 2001; Kokoski et al., 1990).

FDA sets data requirements based on concern levels that are largely based on levels of use in food in concert with chemical structures if the ingredient is structurally similar to a chemical with known toxicity of concern.[12] Detailed guidelines are given by FDA on design and conduct of the study, including number of animals per dose groups, and tissues and fluids to be examined. FDA also requires that the studies are conducted according to Good Laboratory Practice regulations.[13] These criteria are fairly conservative; except in the most trivial exposure situations, most new ingredients require a set of chronic and developmental toxicity studies, as well as a full battery of short-term studies for mutagenicity and genotoxicity. In these cases, FDA uses a 100-fold safety factor to calculate the ADI from the NOAEL. If only subchronic studies are available, FDA uses an additional uncertainty factor of ten, which translates to a safety factor of 1,000 (Frankos and Rodricks, 2001; Kokoski et al., 1990; Lu, 1988).

This methodology for setting an ADI has its limitations. The methodology cannot be used where estimated consumption exceeds 1.5 grams per person per day because practical limitations preclude feeding rodents sufficiently high levels to achieve a margin of safety of 100-fold. In these cases, it has been suggested that there be an absence of adverse effects at doses approaching 2500 mg/kg bw per day, which is viewed as a practical limit in rodents (Borzelleca, 1992). In these instances, the safety evaluation needs to rely on scientific judgment from a variety of studies. In general, there needs to be a high NOAEL with lack of serious findings in the animal toxicology studies coupled with clean clinical studies in humans at the proposed use levels or good arguments based on ADME considerations or background occurrence in the diet.

[12] See a useful summary of FDA requirements by exposure level and chemical structure in FDA guidelines at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm054658.htm Accessed November 5, 2017.

[13] See https://www.fda.gov/ICECI/Inspections/NonclinicalLaboratoriesInspectedunderGoodLaboratoryPractices/default.htm. Accessed November 2, 2018

FDA does not rigidly adhere to these guidelines for testing requirements in all cases. For purified natural extracts or natural products where the biological source is a common food, or the source is not of concern to FDA, the agency will usually agree with GRAS determinations for use at dietary levels of the extract or natural product that are equivalent to the average exposure to natural sources in the diet without requiring any new or additional toxicity data. However, if data in the literature indicate possible adverse effects, FDA will normally insist that more studies are undertaken to investigate the safety of the ingredient. Additional studies are normally required for allowance of higher use levels.

E. Common Knowledge Elements for GRAS Conclusions

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS conclusion requires that consensus exists within the broader scientific community.

1. Public Availability of Scientific Information

Relevant studies of NaHA and RCE, including *in vitro* mutagenicity studies, animal toxicology studies and human clinical trials have been published in the scientific literature. A number of unpublished *in vitro*, animal and human studies conducted by Bloomage provide corroborative evidence that Bloomage's manufacturing process and extended molecular weight range do not change the safety profile of the material. One previous GRN, (GRN No. 491), for Bioiberica S.A.'s rooster combs extract that contains 60-80% NaHA is available on the FDA's GRAS Notice Inventory website (Bioiberica S.A., 2014). This GRAS evaluation satisfies the first common knowledge element, as the scientific information that is the basis of the GRAS determination for NaHA is publicly available.

2. Scientific Consensus

The second common knowledge element for a GRAS conclusion requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use. Bloomage intends to add its NaHA of MW 10-4000 kDa that is produced by bacterial fermentation as an ingredient in beverages, including fruit drinks/ades and carbonated soft drinks, candy, milk and milk products, and ready-to-eat cereals. Bloomage estimates the mean and 90th percentile estimated dietary intakes at the proposed maximum intended use levels of NaHA to be 125 and 250 mg NaHA/day, respectively. This dose does not present a safety concern to humans.

One notification related to NaHA was identified in a search of FDA's GRAS Notice Inventory website. Bioiberica S.A.'s GRN No. 491 for the use of Rooster Combs Extract (RCE) (60-80% HA) as an ingredient in food at 80 mg/serving and up to 2 servings per day received a "no questions" response from FDA (Bioiberica S.A., 2014). No GRNs for sodium hyaluronate or hyaluronic acid were identified. In May 2013, EFSA adopted a Scientific Opinion that concluded Bioiberica's RCE is safe under the proposed use in dairy products at a maximum dose of 80 mg/day (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013) and RCE was subsequently placed on the European market as a novel food ingredient under regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2013) 8319) (2013/705/EU)). In 1996, the Japanese Ministry of Health, Labour and Welfare evaluated the safety of HA extracted from rooster combs or obtained by fermentation with *Streptococcus zooepidemicus*. HA was deemed safe for food use and was subsequently listed in the Japanese Food Additives Catalog (Japanese Ministry of Health Labour and

Welfare, 1996). In 2011, the Japanese Health Food and Nutrition Food Association issued a food industry standard for HA, specifying the production and analytical methods for HA and affirming the safety of HA in foods, setting a maximum daily intake of 250 mg per person (Japanese Health Food and Nutrition Food Association, 2011). In 2008, the Ministry of Heath of the People's Republic of China approved HA for use in foods and as a new resource for use in health foods at a daily dose not exceeding 200 mg per person (Ministry of Health of the People's Republic of China, 2008). In Korea, HA is approved as a food additive by its Ministry of Food and Drug Safety (Korean Ministry of Food and Drug Safety, 2014).

Four published (Zuo et al., 2008; Morita, 1991; Wakisaka et al., 1991; Akasaka et al., 1988) and 3 unpublished (Shandong Center for Disease Control and Prevention, 2004; Bloomage Freda Biopharm; Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b) acute toxicity studies in mice and rats were identified that evaluated the oral intake of NaHA produced by bacterial fermentation. Two additional acute toxicity studies in rats were also identified that evaluated the oral intake of RCE or chicken sternal cartilage extract containing 10-80% HA (Canut et al., 2012; Schauss et al., 2007). The highest published LD_{50} of NaHA was 15,000 mg NaHA/kg bw (Zuo et al., 2008).

Two published 90-day feeding studies of NaHA produced by bacterial fermentation were identified in the literature (Zuo et al., 2008; Ishihara et al., 1996). Both 30-day and 90-day unpublished repeated dose oral toxicity studies of NaHA were provided by Bloomage (Shandong Center for Disease Control and Prevention, 2004; Institute for Nutrition and Food Safety Chinese Center for Disease Control and Prevention, 2007b). Three additional published repeated-dose oral studies of RCE or chicken sternal cartilage extract were also identified (Bioiberica, 2010; Canut et al., 2012; Schauss et al., 2007). No chronic studies exceeding 90-days were identified. Canut (2012) is the critical study to support the maximum use limit of NaHA in conventional foods. The NOAEL for RCE obtained in this study is 600 mg/kg bw/day. This RCE contains 60-80% NaHA that allows for calculation of a NOAEL of 360 mg NaHA/kg bw/day in rats. A maximum use level of 250 mg NaHA/day in a 70 kg human (3.6 mg NaHA/kg bw/day) provides a 100x safety factor between the dose in humans and the NOAEL of 360 mg NaHA/kg bw/day in rats.

Two published (Tashiro et al., 2012; Bellar et al., 2019) and one unpublished (Hunan Provincial Center for Disease Control and Prevention, 2012) human studies that evaluated the oral intake of NaHA (MW 35-900 kDa) produced by bacterial fermentation were identified. Four additional studies evaluating the oral intake of RCE containing HA were also considered (Kalman et al., 2008; Martinez-Puig et al., 2013; Sola et al., 2015; Nagaoka et al., 2010). NaHA doses of 100-200 mg/day were tolerable and without adverse effects.

Numerous *in vitro* safety studies, including bacterial reverse mutation assays, mouse micronucleus testing, sperm malformation testing and chromosomal aberration assays did not reveal any mutagenic potential of NaHA or RCE.

Bloomage and the Expert Panel maintain that well-qualified scientists would conclude that Bloomage's NaHA is generally recognized as safe for use in food given the regulatory and safety data available and using well accepted toxicological principles.

F. Conclusion

In consideration of the aggregate safety information available on Bloomage's NaHA, Bloomage and the Expert Panel conclude that Bloomage's NaHA, when consumed in foods as described within this GRAS Conclusion, is generally recognized as safe (GRAS) within the meaning of the Food, Drug, and Cosmetic Act.

Bloomage's NaHA, when produced under ISO9001:2015/ISO22000:2005 and in accordance with FDA Good Manufacturing Practices requirements and when it meets those specifications presented by Bloomage in Table 1 is Generally Recognized As Safe when consumed at the levels and uses described herein. The quantity of a substance added to food should not exceed the amount reasonably required to accomplish its intended technical effect.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

[redacted]

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Chair

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PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE

A. List of Acronyms

bw	Body weight
BP	Blood pressure
BUN	Blood Urea Nitrogen
C°	Celsius
CFR	Code of Federal Regulations
cfu/g	Colony-forming Unit/gram
CIR	Cosmetic Ingredient Review
COA	Certificate of Analysis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EC	European Commission
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HA	Hyaluronic Acid
HAS	Hyaluronan Synthase
HR	Heart Rate
hsCRP	High-sensitivity C-reactive Protein
IL-6	Interleukin-6
IR	Infrared
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
LD_{50}	Median Lethal Dose
LLC	Limited Liability Corporation
µg	Microgram
mg	Milligram
ml	Milliliter
MW	Molecular weight
NaHA	Sodium hyaluronate
ND	Not detected
NHANES	National Health and Nutrition Examination Survey
NLT	Not Less Than
NMT	Not More Than
NS	Not stated
NOAEL	No-observed-adverse-effect-level
NOEL	No-effect-level
NR	Not reported
OECD	Organization for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
PL	Phospholipid
ppm	Parts per million
RBC	Red Blood Cell
RCE	Rooster Combs Extract
RHAMM	Receptor for HA-mediated Motility
TNF-α	Tumor Necrosis Factor α

USP	United States Pharmacopeia
WBC	White Blood Cell
w/w	Weight/Weight

B. References

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C. Appendices

Appendix 1 Expert Panel Report of the GRAS Status of Bloomage Biotechnology Corporation's NaHA

Introduction

Bloomage Biotechnology Corp., Ltd. ("Bloomage"), has determined that their Sodium Hyaluronate (NaHA) is "Generally Recognized As Safe", using scientific procedures in accordance with Section 201 (s) of the Federal Food, Drug and Cosmetics Act. Bloomage intends to use NaHA (MW 10-4000 kDa) as an ingredient in food and beverages, including fruit drinks/ades and carbonated soft drinks, milk and milk products, and ready-to-eat cereals. The addition of NaHA to these products is intended for those individuals wishing to increase their daily intake of HA. Bloomage estimates the mean and 90th percentile estimated dietary intakes at the proposed maximum intended use levels of NaHA to be 125 and 250 mg NaHA/day, respectively.

At the request of Bloomage, GRAS Associates, LLC, assembled a panel of experts (GRAS Panel) who are qualified by scientific training and experience to conduct an independent evaluation of the safety and GRAS status of Bloomage's NaHA when used as proposed by Bloomage in the accompanying GRAS dossier. The GRAS Panel's approach to the evaluation and the Panel's findings are described below.

Discussion

The GRAS Panel reviewed information about the manufacturing, chemical composition and specifications for NaHA produced from microbial fermentation, the history of use of NaHA and related substances, the estimated exposure to NaHA and its metabolite, N-acetylglucosamine, resulting from the proposed use of NaHA, and the stability and safety/toxicity of NaHA.

Bloomage manufactures NaHA using food grade chemicals under ISO9001:2015 and ISO22000:2005 regulations and in compliance with FDA current Good Manufacturing Practices. The GRAS Panel reviewed data provided by Bloomage that show that six non-consecutive batches of NaHA meet Bloomage's specifications. Bloomage has shown that NaHA is stable under conditions of accelerated stability for 6 months at 45˚C and 75% relative humidity and for 36 months under ambient conditions at 25˚C and 60% RH. These data support Bloomage's conclusion that their NaHA is shelf-stable for 36 months.

The US regulatory history for NaHA includes, Food Chemicals Codex (FCC) specifications for NaHA produced from microbial fermentation (Food Chemicals Codex, 2018) and one GRAS notification for the use of Rooster Combs Extract (RCE) as an ingredient in food that received a "no questions" response from FDA (Bioiberica S.A., 2014). Bioiberica's RCE (IB0004) consists of approximately 60-80% NaHA, 20% glycosaminoglycans and 20% partially hydrolyzed proteins. No GRNs for NaHA or HA were identified.

In the European Union, EFSA adopted a Scientific Opinion that concluded Bioiberica's RCE is safe under the proposed use in dairy products at a maximum dose of 80 mg/day (EFSA Panel on Dietetic Products Nutrition and Allergies, 2013) and RCE was subsequently placed on the European market as a novel food ingredient under regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2013) 8319) (2013/705/EU)). NaHA has been approved for use as a food additive in Japan (Japanese Ministry of Health Labour and Welfare, 1996). The Japanese

Heath Food and Nutrition Food Association issued a food industry standard for HA and affirmed the safety of HA in foods, setting a maximum daily intake of 250 mg per person (Japanese Health Food and Nutrition Food Association, 2011). In Korea, HA is approved as a food additive by its Ministry of Food and Drug Safety (Korean Ministry of Food and Drug Safety, 2014). In 2008, the Ministry of Heath of the People's Republic of China approved HA for use in foods and as a new resource for use in health foods at a daily dose not exceeding 200 mg per person (Ministry of Health of the People's Republic of China, 2008). In Canada, HA (purified or from bacterial fermentation) is classified as a Natural Health Product under Schedule 1, item 2 (an isolate) of the Natural Health Products regulations.

The GRAS Panel reviewed information regarding the absorption, distribution, metabolism and excretion of NaHA. Numerous studies have investigated the metabolism and safety of NaHA and HA from several sources by oral and other routes of administration. Several unpublished in vitro, animal and human studies were conducted by Bloomage and provide corroborative evidence that Bloomage's manufacturing process and extended molecular weight range do not change the safety profile of the material.

- Neither NaHA nor RCE is mutagenic in Salmonella test strains, mouse micronucleus testing, sperm malformation testing or chromosomal aberration assays.
- Acute oral toxicity studies provided the highest published LD50 of NaHA as 15,000 mg/kg bw (Zuo et al., 2008).
- A 30-day repeated oral toxicity study in rats reported no toxicity at a dose of 1,500 mg/kg bw/day, the highest dose tested (Shandong Center for Disease Control and Prevention, 2004).
- A 90-day feeding study of Bioiberica's RCE is the critical study to support the maximum use limit of NaHA in conventional foods (Canut et al., 2012). The NOAEL for RCE obtained in this study is 600 mg/kg bw/day. This RCE contains 60-80% NaHA that allows for calculation of a NOAEL of 360 mg NaHA/kg bw/day in rats. A maximum use level of 250 mg NaHA/day in a 70 kg human (3.6 mg NaHA/kg bw/day) provides a 100x safety factor between the dose in humans and the NOAEL of 360 mg NaHA/kg bw/day in rats.
- No chronic studies exceeding 90-days were identified.
- Two published (Tashiro et al., 2012; Bellar et al., 2019) and one unpublished (Hunan Provincial Center for Disease Control and Prevention, 2012) human studies evaluated the oral intake of NaHA (MW 35-900 kDa) produced by bacterial fermentation. NaHA doses of 100-200 mg/day were tolerable and without adverse effects.
- A comprehensive review of the reproductive toxicity of NaHA, concluded that hyaluronic acid is a naturally occurring glycosaminoglycan and is not expected to adversely affect pregnancy outcome (REPROTOX, 2018). This conclusion was based on no adverse outcomes in rat and rabbit fertility and pregnancy studies at doses ranging from 40-64 mg/kg/day; the normal presence of HA in female reproductive tract fluids as well as being a constituent of the cumulus matrix, zona pellucida and perivitelline space; and the role that HA appears to play in oocyte maturation and cumulus expansion and its ability to promote sperm function including motility, capacitation and acrosome reaction.
- The causal role of HA in tumor formation, progression and metastasis was extensively reviewed by the Cosmetic Ingredient Review (CIR) Expert Panel that concluded "that HA likely

does not play a causal role in metastasis and that increased expression of HA genes may be a consequence of metastatic growth not the converse (Becker et al., 2009)."

- NaHA and HA are used in dietary supplements in the US with no evidence of adverse effects further supporting the safety of this ingredient.

Although numerical estimates of background dietary intake of NaHA or HA were not identified, data on blood and tissue concentrations in several animals are available. Serum or plasma concentrations in sheep and pigs range from 100 to 260 ng/ml (Lebel, 1991) and the concentration of HA in skeletal muscle of rabbits is reported to be 26 to 28 μg/g (Necas et al., 2008). The concentration of HA in the skin of rabbits is much higher at 840 μg/g. Humans are exposed to endogenous background levels due to intracellular HA synthesis in the Golgi network by the integral membrane proteins, hyaluronan synthases. The total amount of HA in the human body is estimated at 14-16 g with half of that located in the skin (Becker et al., 2009). Normal concentrations in the plasma of healthy human volunteers are much lower with a range of 10-100 ng/ml and a mean value of 30-40 ng/ml (Lebel, 1991). The normal daily turnover of HA in humans is 34 mg/day.

Conclusion

The GRAS Panel critically reviewed the information provided by Bloomage as well as other publicly available information. The GRAS Panel agrees that the data and information relied upon by Bloomage to establish safety are generally available and, therefore, satisfy the first common knowledge element for a GRAS conclusion. The GRAS Panel agrees that the second common knowledge element is met, in that consensus exists within the broader scientific community about the safety of NaHA.

The Expert Panel has concluded that under the proposed conditions of use listed above, Bloomage's NaHA is GRAS by scientific procedures in that there is a "reasonable certainty of no harm under the intended condition of use" when manufactured as described using cGMPs.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that NaHA, when used as described, is GRAS.

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Appendix 2 Identification and 16 S rDNA Sequence Determination of Bloomage's *Streptococcus zooepidemicus* Parental Strain

The Institute of Microbiology, Chinese Academy of Sciences

Test and Identification Report

Department of delivery: Shandong Freda Biochem Co., Ltd.
Test substance: Strain on slope (frd)
Quantity of sample: One Date of acceptance: October, 2005

Person responsible for test and identification: Zhou Yu-guang Operator: Liu Ying-hao

Results:

Results of physiological and biochemical tests:

Items	Result	Items	Result
Gram staining	Positive	Acid from:	
Cell shape	Sphere, ellipse	Fructose	+
Contact enzyme	-	Ribose	+
Oxidase	-	Raffinose	-
Growth in air	+	Lactose	+
Growth anaerobically	+	Melizitose	-
O/F test	Fermenting	Salicin	+
Production of lactic acid	+	Rhamnose	-
Aerogenesis from:		Esculine	-
Glucose	-	Mannose	+
Sodium gluconate	-	Mannitol	-
Acid from:		Maltose	+
Glucose	+	Sorbitol	+
Sodium gluconate	-	Melibiose	-
Cellobiose	-	Galactose	+
Arabinose	-	Growth at 45℃	-
Sucrose	+	Growth at 10℃	-
Trehalose	-	Growth at pH 9.6	-
Xylose	-	Growth with 6.5% NaCl	-

Data for Bloomage's *Streptococcus zooepidemicus* parental strain (English translation)

The Institute of Microbiology, Chinese Academy of Sciences

Test and Identification Report

Department of delivery: Shandong Freda Biochem Co., Ltd.
Test substance: Strain on slope (frd)
Quantity of sample: One Date of acceptance: October, 2005

Person responsible for test and identification: Zhou Yu-guang Operator: Liu Ying-hao

---

Results:

Results of sequence determination of 16S rDNA:

CGCACAGATGATACGTAGCTTGCTACAATTATCTGTGAGTCGCGAACGGGTGAGTAACG
CGTAGGTAACCTAGCTTATAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCAT
AAAAGTGGTTGACCCATGTTAACCATTTAAAAGGAGCAACAGCTCCACTATGAGATGG
ACCTGCGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATACATAGC
CGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGACCGAGCAACGCCGCG
TGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGAACAGTGATGGG
AGTGGAAAGTCCATCATGTGACGGTAACTAACCAGAAAGGGACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCAGTGGCTTAACCATTGTATGCTTT
GGAAACTGTTAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTG
ACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGCTGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGTAGCTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCCGATGCTATTCTTAGAGATAAGAAGTTACTTCGGTACATTG
GAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GC

Identification result: *Streptococcus equi* subsp. *Zooepidemicus*

The Institute of Microbiology, Chinese Academy of Sciences
(Seal)
January 20, 2006

Data for Bloomage's *Streptococcus zooepidemicus* parental strain (English translation)

中国科学院微生物研究所

检测鉴定报告

(2006)微检字第007号
共 2 页第 01 页

送检单位：山东福瑞达生物化工有限公司
样品名称：斜面菌种（frd）
样品数量：1 株　　送检时间：2005 年 10 月
检测鉴定负责人（签字）：周宇元　　检测鉴定操作人（签字）：刘英云

检测鉴定内容、结果：(本鉴定结果仅对送检样品有效，未经鉴定方同意，鉴定结果不得用于商业宣传)

生理生化实验结果：

试验项目	结果	试验项目	结果
革兰氏染色	阳性	碳水化合物产酸（续）	
细胞形状	球形、椭圆	果　糖	+
接触酶	−	核　糖	+
氧化酶	−	棉子糖	−
在空气中生长	+	乳　糖	+
兼性厌氧生长	+	松三糖	−
O/F 试验	发酵	水杨素	+
产生乳酸	+	鼠李糖	−
碳水化合物产气		七叶灵	−
葡萄糖	−	甘露糖	+
葡萄糖酸钠	−	甘露醇	−
碳水化合物产酸		麦芽糖	+
葡萄糖	+	山梨醇	+
葡萄糖酸钠	−	蜜二糖	−
纤维二糖	−	半乳糖	+
阿拉伯糖	−	45℃生长	−
蔗　糖	+	10℃生长	−
海藻糖	−	pH9.6 生长	−
木　糖	−	6.5%NaCl 生长	−

Data for Bloomage's *Streptococcus zooepidemicus* parental strain (original Chinese)

中国科学院微生物研究所

检 测 鉴 定 报 告

(2006)微检字第 007 号

共 2 页第 01 页

送检单位：山东福瑞达生物化工有限公司

样品名称：冻干菌种（frd）

样品数量：1 株　　送检时间：2005 年 10 月

检测鉴定负责人（签字）：周宇元　　检测鉴定操作人（签字）：

检测鉴定内容、结果：(本鉴定结果仅对送检样品有效。未经同意，不得将鉴定方的名称用于商业宣传)

16S rDNA 序列测定结果

CGCACAGATGATACGTAGCTTGCTACAATTATCTGTGAGTCGCGAACGGGTGAGTAACG
CGTAGGTAACCTAGCTTATAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCAT
AAAAGTGGTTGACCCATGTTAACCATTTAAAAGGAGCAACAGCTCCACTATGAGATGG
ACCTGCGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATACATAGC
CGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGACCGAGCAACGCCGCG
TGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGAGAAGAACAGTGATGGG
AGTGGAAAGTCCATCATGTGACGGTAACTAACCAGAAAGGGACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCAGTGGCTTAACCATTGTATGCTTT
GGAAACTGTTAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTG
ACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGCTGAGTGCTAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGTAGCTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCCGATGCTATTCTTAGAGATAAGAAGTTACTTCGGTACATTG
GAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GC

鉴定结果：***Streptococcus equi*** subsp. ***zooepidemicus*** 马链球菌兽疫亚种

中国科学院微生物研究所
2006 年 01 月 20 日

Data for Bloomage's *Streptococcus zooepidemicus* parental strain (original Chinese)

Appendix 3 Bloomage's Specifications for NaHA (from microbial fermentation)

Items		Acceptance criteria
Character		White or almost white powder or granules
Identification	Infrared absorption	Consistent with reference spectrum of Sodium Hyaluronate
	Reaction of sodium	Positive
pH		6.0~8.0
Loss on drying		≤10.0%
Molecular weight		10k~4000k Da
Ash		≤13.0 %
Lead		≤1 ppm
Arsenic		≤ 2 ppm
Bacteria counts		≤ 500 cfu/g
Mold & Yeast		≤100 cfu/g
Escherichia coli		Negative
Staphylococcus aureus		Negative
Salmonella		Negative
Sodium Hyaluronate		≥93.0 %

METHODS

CHARACTER

Visual, White or almost white powder or granules.

TESTS

Loss on Drying

Place approximate 1.0g of the sample in the plate of Halogen Moisture analyzer, heat to 110℃ and then test for 15 minutes.

Molecular Weight

Test solution

Accurately weigh 100 mg (W_1) of the sample to a 100 ml volumetric flask, add appropriate amount of 0.2mol/L NaCl solution, place the volumetric flask on an oscillator till dissolve and then dilute to 100ml, and mix well; Weigh appropriate amount of the solution (W_2) to a 100ml volume volumetric flask, dilute with 0.2mol/L NaCl solution to 100ml, and mix well, the test solution is obtained.

Filter appropriate amount of 0.2mol/L NaCl solution through a No.3 sintered -glass filter and discard the primary filtration. Determine the flow-time for 0.2mol/L NaCl solution (t_0) with ubbelohde viscometer (Internal diameter of tube 0.53mm).

Filter appropriate amount of test solution through a No.3 sintered -glass filter and discard the primary filtration. Determine the flow-time for test solution (t_1) with ubbelohde viscometer (Internal diameter of tube R 0.53mm).

The intrinsic viscosity is calculated according to the following formula (1):

Intrinsic Viscosity: $[\eta] = \frac{\sqrt{2[(\frac{t_1}{t_0}-1)-ln\frac{t_1}{t_0}]}}{C}$ (1)

In which,

t_0: flow-time for 0.2mol/L NaCl solution, second

t_1: flow-time for test solution, second

c—concentration of test solution, g/dL.

The molecular weight is calculated according to the following formula (2):

Molecular Weight: $Mr=[\frac{[\eta]\times 10^5}{36}]^{\frac{1}{0.78}}$ (2)

Ash

Accurately weigh about 1.0g of the sample in a crucible of constant weight, and ignite slowly until the substance is completely carbonized, gradually heat to 700 to 800 ºC for 1.5h until completely ashed and constant weight.

Lead

Weigh about 0.25g of test sample in a 25ml volumetric flask, add 1ml of nitric acid, 1ml of 30% hydrogen peroxide, and place the volumetric flask in boiling water to dissolve, cool and dilute with water to 25ml. Prepare two parts in parallel.

Lead reference solution is diluted with 1% nitric acid solution to reference series solution with concentration 0, 2.5, 5 10, 20 and 30 ng/ml, respectively.

Use 20g/l of ammonium dihydrogen phosphate solution as matrix modifier, wave length 283.3nm, the absorbance was measured by graphite furnace atomic absorption spectrometry, and the content of lead in the test sample was calculated.

Arsenic

Accurately weigh 1.0 g of the sample to be examined, test according to the first method of the 0822 of the fourth section of the Chinese Pharmacopoeia 2015 edition.

Bacteria counts, Mold & Yeast counts

Take the sample to be examined, test according to the method of the 1105 of the fourth section of the Chinese Pharmacopoeia 2015 edition.

Escherichia coli, Staphylococcus aureus and Salmonella

Take the sample to be examined, test according to the method of the 1106 of the fourth section of the Chinese Pharmacopoeia 2015 edition.

ASSAY

Reference solution: Accurately weigh 0.1g (Ws) of glucuronic acid dried at 105 ^{o}C with phosphorus pentoxide, add water to make 50ug per 1ml solution, and mix well, reference solution is obtained.

Test solution: Accurately weigh 0.1g (W_1) of test sample in a 100ml volumetric flask, add appropriate amount water to dissolve and dilute to 100ml till the test sample completely dissolved, and mix well. Weigh about 4.0g (W_2) (density of solution is considered as 1.000g/ml, i.e. 4ml) of the solution in a 50ml volumetric flask, dilute with water to 50ml, and mix well, test solution is obtained.

Standard curve: Accurately pipette 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the reference solution, place in capped test tubes, add water to 1.0ml, mix well and cool to below 4^{o}C, slowly drip drop 5ml of 0.025 mol/L borax sulfuric acid solution, mix well, heat in boiling water for 10min, and then cool in ice water, respectively. Accurately add 0.20ml of carbazole solution, shake well, heat for 15min in boiling water, and then cool in ice water. Test ultraviolet absorbance with 530nm wavelength, regression equation and correlation coefficient are obtained from the concentration (ug/ml) of glucuronic acid and absorbance.

Test of sample: Accurately pipette 1.0ml of test solution in two capped test tubes, respectively, test according to the same method of 'Stand curve' from description of below 4°C, concentration (Ci) (ug/ml) of glucuronic acid is obtained from regression equation, the assay of sodium hyaluronate is calculated according to dilution factor. Calculate the assay of sodium hyaluronate from the expression:

$$Assay\ (\%) = \frac{\mathrm{Ci} \times 50 \times 100 \times 401.3 \times \rho \times 100}{W_1 \times (100 - \mathrm{h}) \times W_2 \times 10^6 \times 194.1} \times 100\%$$

In which,

Ci: concentration of glucuronic acid, ug/ml

W_1: mass of test sample, g

W_2: mass of dilution solution, g

h (%):loss on drying of test sample

ρ: density of the test solution at 25°C,1.000 g/ml

401.3: molecular weight of disaccharide unit

194.1: Molecular weight of glucuronic acid

Appendix 4 Nutritional Profile for Bloomage's NaHA

eurofins

Bloomage Freda Biopharm Co.,Ltd.

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SU0003914

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Report date:7/18/2014

Print By:Happy Chen

CERTIFICATE OF ANALYSIS

Certificate No.: AR-14-SU-010241-02

This report invalidates all previous versions.

Sample

Client Sample Description Sodium Hyaluronate	Sample Code	50214Q010054
	Date of order	2014.04.14
	Sample received	2014.04.14
	Start of Analysis	2014.04.14
	End of Analysis	2014.07.17
	Reception temperature	18.8℃
	Quantity of Sample	1*170g
	Sample packaging	Sealed plastic bottle(screwed cap)
	Sample appearance	/
Client Sample Code 1403252		

Results and comments are shown on the following page(s)

The result(s) relate(s) only to the item (s) tested.
Eurofins General Terms and Conditions apply.

For and on behalf of
Eurofins Technology Service (Suzhou) Co., Ltd

Kevin Yu
Deputy of Technical Director

eurofins

Results of Analysis

	Results	LOQ	LOD	Unit	Comments
SU05H	Iron (ICP-MS), EN ISO 17294-2 2005 mod., ICP-MS				
Iron (Fe)	1.37	0.1		mg/kg	
SU086	Calcium (ICP-OES), Internal method, ICP-OES				
Calcium (Ca)	127	3		mg/100 g	
SU088	Sodium (ICP-OES), Internal method, ICP-OES				
Sodium (Na)	5800	4.5		mg/100 g	
SU20U	Total fat, AOAC 963.15, Hydrolysis-Soxhlet Extraction Method				
Total fat	<0.1	0.1		g/100 g	
SU20Y	Moisture, AOAC 935.29, Gravimetric				
Moisture	4.30	0.01		g/100 g	
SU21V	Fatty acid profile, EN ISO 15304, GC-FID				
mono-unsaturated fatty acids total	Not Detected	0.1		g/100 g	
poly-unsaturated fatty acids total	Not Detected	0.1		g/100 g	
saturated fatty acids total	Not Detected	0.1		g/100 g	
total of trans fatty acids	Not Detected	0.1		g/100 g	
SU227	Ash, AOAC 941.12, Gravimetric				
Ash	13.9	0.01		g/100 g	
SU348	Cholesterol, AOAC 994.10 mod., GC/MS				
Cholesterol	Not Detected	0.5		mg/100 g	

⋆ means the test is subcontracted within Eurofins group
∘ means the test is subcontracted outside Eurofins group.

Not Detected: Means not detected at or above the Limit of Quantification (LOQ)

END OF REPORT

The Eurofins Ash analysis method is AOAC 941.12 Gravimetric. Bloomage's Ash analysis method follows their enterprise standards. The severity of the conditions differs between the two methods, which results in different ash content.

Appendix 5 Specifications and Certificates of Analysis for Multiple Batches of Bloomage's NaHA

BLOOMAGE FREDA BIOPHARM CO., LTD.

NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS **16-E03140303**

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1603073 Production Date: Mar.07, 2016
Quantity: 191.73 kg Expiry Date: Mar.07, 2019

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.2
Loss on drying	≤10.0%	7.7%
Molecular weight	10K～4000K Da	1370K Da
Ash	≤13.0%	10.3%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	95.5%

Conclusion: up to the standard

Reported by: **Approved by:**

BLOOMAGE FREDA BIOPHARM CO., LTD.

NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS 16-E03280203

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1603218
Quantity: 140 kg

Production Date: Mar.21, 2016
Expiry Date: Mar.21, 2019

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.3
Loss on drying	≤10.0%	7.0%
Molecular weight	10K～4000K Da	510K Da
Ash	≤13.0%	10.1%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	95.4%

Conclusion: up to the standard

Reported by: 张明娟

Approved by:

BLOOMAGE FREDA BIOPHARM CO., LTD.

NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS **17-E06292003**

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1706224
Quantity: 201.2 kg

Production Date: June 22, 2017
Expiry Date: June 22, 2020

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.5
Loss on drying	≤10.0%	6.8%
Molecular weight	10K～4000K Da	1060K Da
Ash	≤13.0%	10.1%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	96.4%

Conclusion: up to the standard

Reported by:

Approved by:

BLOOMAGE FREDA BIOPHARM CO., LTD.

NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS **18-E04081603**

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1803313 Production Date: Mar.31, 2018
Quantity: 243.098 kg Expiry Date: Mar.31, 2021

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.4
Loss on drying	≤10.0%	7.5%
Molecular weight	10K～4000K Da	1320K Da
Ash	≤13.0%	11.9%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	96.0%

Conclusion: up to the standard

Reported by: **Approved by:**

BLOOMAGE FREDA BIOPHARM CO., LTD.
NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS 18-E09261503

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1809191 Production Date: Sept.19, 2018
Quantity: 404.728 kg Expiry Date: Sept.19, 2021

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.4
Loss on drying	≤10.0%	5.2%
Molecular weight	10K～4000K Da	530K Da
Ash	≤13.0%	11.2%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	95.5%

BLOOMAGE FREDA BIOPHARM CO., LTD. 质检专用章 山东福瑞达生物医药有限公司

Conclusion: up to the standard

Reported by: **Approved by:**

BLOOMAGE FREDA BIOPHARM CO., LTD.

NO.678 TIANCHEN ST., HIGH-TECH DEVELOPMENT ZONE,
JINAN, CHINA 250101

CERTIFICATE OF ANALYSIS 19-E03271404

Product Name: Sodium Hyaluronate (Food Grade)
Batch No.: 1903208 Production Date: Mar.20, 2019
Quantity: 114.988 kg Expiry Date: Mar.20, 2022

Item	Standard	Result
[Character]	White or almost white powder or granules	Complied
[Identification]		
Infrared absorption	Consistent with the *reference spectrum of sodium hyaluronate*	Complied
Reaction of sodium	Positive	Positive
[Test]		
pH	6.0～8.0	6.2
Loss on drying	≤10.0%	6.6%
Molecular weight	10K～4000K Da	380K Da
Ash	≤13.0%	9.4%
Lead	≤1 ppm	<1 ppm
Arsenic	≤2 ppm	<2 ppm
Bacteria counts	≤500 cfu/g	Complied
Mold & Yeast	≤100 cfu/g	Complied
Escherichia coli	Negative	Negative
Staphylococcus aureus	Negative	Negative
Salmonella	Negative	Negative
[Assay]		
Sodium hyaluronate	≥93.0%	96.7%

Conclusion: up to the standard

Reported by: **Approved by:**

Appendix 6 Analysis Report for *Streptococcus hemolyticus*

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诺安检测
Sino Analytica

Sino Analytica (Qingdao) Ltd
诺安检测服务(青岛)有限公司
63 Shang He Road, Qingdao 266012, China
中国青岛市商河路63号 邮编266012
T 电话 +86 532 838 16633
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Certificate of Analysis 分析证书 No.LR115143

To 致：Bloomage Freda Biopharm Co., Ltd华熙福瑞达生物医药有限公司
Address 地址: No.678 Tianchen St.,High-Tech Development Zone Jinan Shandong China 中国山东济南高新技术开发区天辰大街678号
Date of Report报告日期 (yyyy/mm/dd): 2013-4-22

Sample Information 样品信息

The following sample was/were supplied by/on behalf of you下列样品是贵公司或以贵公司的名义提供的:

Date of Receipt接样日期(yyyy/mm/dd): 2013/04/12

Sample Description 样品描述	Condition on Receipt 接收时状态	Sample ID 样品编号
透明质酸钠 1209242	Dehydrated/脱水, 210g, Plastic bottle packaged/塑料瓶包装, By express/快递	LR115143-001

The results apply only to the samples as received, and shall not be used for undue propaganda without written approval of Sino Analytica. 本结果仅适用于来样。未经诺安书面批准，不得用于不当宣传。

Edited By 编制人：Mavis Ju 鞠洪娣

Authorised Signatory授权签字人：

SINO ANALYTICA (QINGDAO) 诺安检测服务(青岛)有限公司 报告专用章 STAMP FOR REPORT ONLY

Meng Fu付萌
Lab Manager实验室经理

诺 安 检 测
Sino Analytica

Sino Analytica (Qingdao) Ltd
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Requested Test(s) 检测要求

Sample ID 样品编号	Requested Tests 检测项目	Sample Preparation 样品预处理
LR115143-001	Arsenic/砷	Original sample 原样
	Lead /铅	Original sample 原样
	Mercury/汞	Original sample 原样
	Aerobic Colony Count / 菌落总数	Original sample 原样
	Coliforms / 大肠菌群	Original sample 原样
	Mould & Yeast / 霉菌与酵母菌	Original sample 原样
	S.aureus / 金黄色葡萄球菌	Original sample 原样
	Salmonella 25g/沙门氏菌25g	Original sample 原样
	Shigella / 志贺氏菌	Original sample 原样
	Streptococcus hemolyticus /溶血性链球菌	Original sample 原样
	Ash/灰分	Original sample 原样
	Moisture / 水分	Original sample 原样

Results 结果

Start Test Date 检测开始日期：2013-4-16 End Test Date 检测结束日期：2013-4-22

Sections 部门	Analytes分析物(Units单位)	Methods 方法	Rpt Lmt 报告限	Results 结果
Sample ID 样品编号：LR115143-001				
QPC	Arsenic 砷(mg/kg)	GB/T 5009.11-2003 第一法	0.02	< 0.02
QPC	Lead 铅(mg/kg)	GB 5009.12-2010 第一法	0.05	< 0.05
QPC	Mercury 汞(mg/kg)	GB/T 5009.17-2003 第一法	0.005	< 0.005
NMC	Aerobic colony count 菌落总数(cfu/g)	GB 4789.2-2010	---	<10
NMC	Coliforms 大肠菌群(MPN/g)	GB 4789.3-2010 (第一法)	---	<3.0
NMC	Mould 霉菌(cfu/g)	GB 4789.15-2010	---	<10
NMC	Yeast 酵母菌(cfu/g)	GB 4789.15-2010	---	<10
NMC	S.aureus 金黄色葡萄球菌(/25g)	GB4789.10-2010(第一法)	---	Not detected 未检出
NMC	Salmonella 沙门氏菌(/25g)	GB 4789.4-2010	---	Not detected 未检出
NMC	Shigella 志贺氏菌(/25g)	GB 4789.5-2012	---	Not detected 未检出
NMC	Streptococcus hemolyticus 溶血性链球菌(/0.5g)	GB/T 4789.11-2003	---	Not detected 未检出
QPC	Ash 灰分(g/100g)	GB 5009.4-2010	0.1	11.9

诺安检测
Sino Analytica

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QPC	Moisture 水分(g/100g)	GB 5009.3-2010	0.1	7.45

----------End of Report 报告结束----------

Appendix 7 Sterilization experiment of ethanol on *Streptococcus equi* subsp. *Zooepidemicus*

华熙生物 BLOOMAGE BIOTECH

EXPERIMENTAL REPORT

QR 09-006-A

Experiment Name	Sterilization experiment of ethanol on Streptococcus equi subsp. Zooepidemicus	Project No.	---
Date	2020.05.18~2020.05.21	Report date	2020.05.21
Reporter	Shanshan Wang	Reviewer	Liping Qiao

Aim

To investigate the sterilization effect of the ethanol precipitation on the production strain Streptococcus equi subsp. Zooepidemicus

Design

Take HA fermentation broth and precipitate it with 50-80% of ethanol, take the precipitate and dissolve it in sterile water, and then coat on the plate to count the colonies.

Materials and apparatus

1. Materials
HA fermentation broth, 95% ethanol, sterilized water
2. Apparatus
Pipette, schott flask, rotor, 500mL centrifuge tube, coating rod, etc., sterilized at 121 ° C for 20min for standby;
Shaker, sterile petri dish, etc.

Method and procedure

1. Add ethanol to be precipitated
Separately take 100ml of fermentation broth into two schott flasks (marked with 1# and 2#), add 95% ethanol in to 1# and 2# schott flask under stirring till ethanol concentration to 50% and 75%, stir for 4h.
2. Centrifugation
Pour the mixed solution in the Schott bottle into 1 # and 2 # centrifuge tubes and centrifuge at 10000 rpm for 10 min.
3. Dissolution
Discard the ethanol supernatant of the 1 # and 2 # centrifuge tubes, and then add 50 mL of sterile water to re-dissolve, the suspension is labeled as sample 1, sample 2 respectively.
4. Coating
Pipette from sample 1 and sample 2 and separately coat on 10 plates, each plate was coated with 0.2 mL of suspension. After coating, it was placed in a 37 ° C incubator.
5. Observation and counting
Incubate for 24 hours and count.

华熙生物
BLOOMAGE BIOTECH

Results and analysis

1. Results

Sample 1 and sample 2 were coated on 10 plates, respectively, and no colonies grew (Figure 1 and Figure 2).

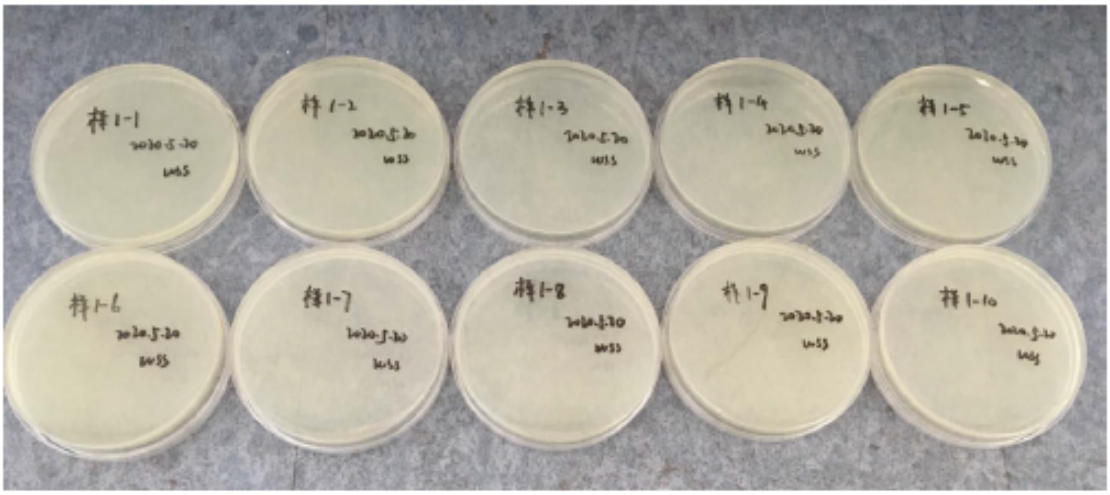

Figure 1 Results of sample 1 after 24 hours of incubation

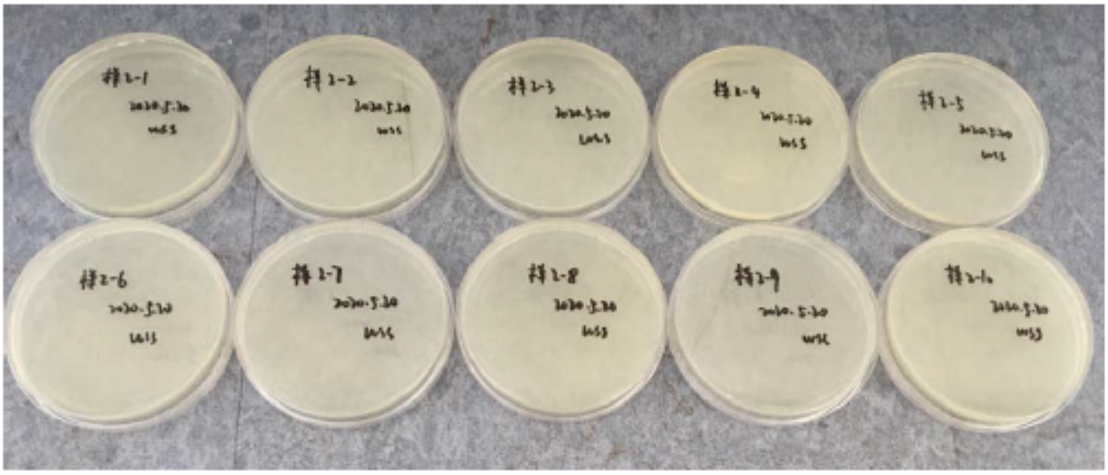

Figure 2 Results of sample 2 after 24 hours of incubation

2. Analysis

Samples 1 and 2 showed no colony growth after 24 hours of incubation after coating, indicating that 50% and 75% ethanol concentration can completely kill the production strain, that is, the ethanol precipitation step in the production process can completely kill the production strain.