Anti-inflammatory and regenerative effects of MKARE® Eggshell Membrane: An *in vitro* osteoarthritis model and placebo-controlled clinical study

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ABSTRACT

MKARE®, a 100% natural ingredient derived from fresh eggshell membrane (ESM), has a rich composition in bioactive compounds like collagen, hyaluronic acid, and elastin. These components are beneficial for managing osteoarthritis (OA) due to their anti-inflammatory and regenerative properties. Highlighting the significance of freshness, our research has shown that the effectiveness of MKARE® is higher than that of other commercial products based on ESM that have been stored for several days at room temperature, losing their bioactive compounds. This study explores the MKARE® anti-inflammatory capacity through an *in vitro* and clinical analyses, demonstrating its ability to alleviate OA symptoms and improve joint health. This underscores the crucial role of freshness in optimizing the therapeutic benefits.

1. Introduction

In recent years, the pursuit of natural and sustainable therapeutic agents has prompted the investigation of diverse sources with potential medical applications (Atanasov et al., 2021; Dzobo, 2022; K J Ruff et al., 2009). One particularly promising candidate is the eggshell membrane (ESM), known for its rich composition of bioactive compounds that exhibit anti-inflammatory and regenerative properties. Within the realm of musculoskeletal disorders, especially osteoarthritis (OA), a prevalent inflammatory joint disease impacting millions globally, there has been an escalating demand for innovative interventions (K J Ruff et al., 2009).

Articular cartilage, the specialized tissue covering the ends of bones within joints, plays a crucial role in preserving joint function and facilitating smooth movement (Sophia Fox et al., 2009). Chondrocytes, the primary cellular components of articular cartilage, are responsible for synthesizing and maintaining the extracellular matrix, vital for the integrity and mechanical properties of the cartilage. However, during the progression of OA, an imbalance between pro-inflammatory and anti-inflammatory factors disrupts chondrocyte homeostasis, leading to an increase in pro-inflammatory cytokines, catabolic enzymes, and matrix metalloproteinases (Loeser et al., 2012). Ultimately, this cascade results in cartilage degradation and joint dysfunction (Fernandes et al., 2002).

Due to the constrained regenerative ability of cartilage, the development of therapies capable of mitigating inflammation and promoting chondrocyte regeneration is of great importance in OA management. This has driven researchers to explore natural sources, such as the ESM, for its potential to alleviate inflammation and facilitate tissue repair (Cánovas et al., 2022).

The ESM is a thin, yet complex, biocompatible layer that separates the eggshell (ES) from the egg white. It consists of diverse bioactive components, such as aggrecan (ACAN) collagen, glycosaminoglycans, elastin, and growth factors (Han et al., 2023b). The ESM has demonstrated significant therapeutic potential in wound healing and tissue repair. Due to its multifaceted composition (Han et al., 2023b), ESM emerges as a compelling candidate for addressing inflammatory

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processes linked to OA and promoting the restoration of chondrocyte function (Kiers & Bult, 2021).

Collagen and ACAN, key constituents of the ESM, play a pivotal role in offering structural integrity and support to the extracellular matrix. These contribute to the stability of tissues and their ability to withstand mechanical stress. Additionally, the presence of glycosaminoglycans, such as chondroitin sulfate and hyaluronic acid, suggests a potential role in enhancing joint lubrication and reducing friction, thus alleviating pain and discomfort experienced by OA patients (Dudhia, 2005; Monfort et al., 2008).

Moreover, the ESM contains elastin, a protein that imparts elasticity to tissues, allowing them to withstand repetitive stresses. In the context of OA, regenerative potential of elastin may contribute to the restoration of damaged cartilage and the prevention of further degradation (Li et al., 2021). Another crucial aspect of the ESM membrane is the presence of growth factors, such as transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF). These growth factors have been implicated in tissue repair and regeneration by stimulating cell proliferation, migration, and synthesis of extracellular matrix components. In an inflamed chondrocyte microenvironment, the application of ESM-derived growth factors may counteract the detrimental effects of pro-inflammatory mediators and foster a more conducive environment for tissue healing (Furukawa et al., 2021).

Products based on ESM are currently available on the market, specifically designed for the treatment of OA. These products are commonly promoted as supplements aimed at alleviating joint pain and enhancing mobility (Eggshell Membrane Market Size | Industry Report, 2021–2028, 2023). They are available in various forms, including capsules, tablets, and powders. The ESM market has grown significantly in the last years. This growth is attributed to the increasing demand for nutritional supplements and the adoption of this product in the pharmaceutical, cosmetic, and food and beverage industries (Eggshell Membrane Market Size, Share, Growth 2024–2032, 2023).

MKARE® is a 100 % natural functional ingredient based on fresh ESM, which natively contains a unique source of bioactive compounds with multiple clinically proven health benefits. MKARE® is rich in collagen (I, V and X), hyaluronic acid, elastin, chondroitin sulfate, glucosamine and more than 400 proteins, a perfect matrix of key biomolecules in a single ingredient (Dar et al., 2017). Recent studies emphasize the significance of using fresh eggshell membrane, as it guarantees the maximal preservation of those bioactive compounds (Baláž et al., 2021; Han et al., 2023a; Kiers & Bult, 2021).

The aim of this work was to evaluate the anti-inflammatory potential and regenerative effects of the MKARE® product. For that purpose, we employed a well-established *in vitro* model of chondrocyte inflammation. This model allowed us to simulate the inflammatory processes observed in OA and assess the response of chondrocytes to ESM treatment. Through comprehensive experimental analyses, we aim to elucidate the underlying molecular mechanisms behind the beneficial effects of ESM on chondrocytes. Additionally, we have conducted a clinical study involving patients afflicted with OA, obtaining promising results. Furthermore, we assessed the significance of the freshness of the membrane compared to other products available on the market, particularly those derived from ES that have been stored for several days at room temperature (RT:20–25 °C).

2. Materials and methods

2.1. Obtention and preparation of ES and ESM under different storage conditions

Complete ES and ESM obtained from Arandovo® facilities were analyzed. For this purpose, soluble components of both ES and ESM were solubilized. For the ES study: 200 g of ES generated on the day of analysis were taken, without storage (Fresh ES labeled as ES-F). Additionally, 1 kg of ES was divided into two parts: one part was frozen at

−20 °C for 13 days (frozen ES labeled as ES-Fr) and the other part was stored at room temperature (RT) for 13 days (ES stored at RT for 13 days labeled as ES-RT) until the day of experiment.

For the eggshell membrane (ESM) study: Fresh ESM labeled as MKARE® was obtained from fresh ES (ES-F), and ESM labeled as ESM-RT was obtained from ES stored at RT for 13 days labeled as ES-RT. Both powders had a particle size of 300 μm and a humidity under 5 %.

2.1.1. Preparation of water-soluble fractions of ES

50 g of finely powdered sample (particle size less than 300 μm) was resuspended in 50 mL of milli-Q water with the aim of extracting soluble proteins from ES. The mixture was homogenized and stirred at room temperature for 1 h. Samples were centrifuged at 9,250 g for 10 min, and the supernatant was collected for assays. This process was repeated in triplicate for each sample type. Three different conditions of ES were analyzed: (i) Fresh ES (the same day of the experiment) labeled as ES-F; (ii) frozen (13 days stored at −20 °C) ES labeled as ES-Fr; (iii) ES stored at RT for 13 days labeled as ES-RT.

2.1.2. Preparation of water-soluble fractions of ESM

5 g of the powder from each sample (particle size less than 300 μm) was resuspended in 30 mL of milli-Q water. The mixture was homogenized and stirred at room temperature for 1 h. Samples were centrifuged at 9,000g for 10 min, and the supernatant was collected for assays. This procedure was also performed in triplicate for each sample. Two different conditions were analyzed: (i) Fresh ESM labeled as MKARE®; (ii) ESM stored at RT for 13 days labeled as ESM-RT.

2.2. Evaluation of ES and ESM protein quality

2.2.1. Determination of free amino groups (reaction with OPA)

Total protein content of the supernatants was quantified using Kjeldahl method (Varelis, 2016). Degree of hydrolysis of the water-soluble fractions was estimated by reacting the free amino acid groups with o-phthalaldehyde (OPA), following the method described by (Nielsen et al., 2001) analysis was conducted in triplicate for each soluble fraction obtained.

2.2.2. SDS-PAGE profile analysis

Samples were diluted to a protein concentration of 0.8 mg/mL in treatment buffer containing Tris-HCl (0.05 M, pH 6.8), sodium dodecyl sulfate (SDS, 1.6 % w/v), glycerol (8 % v/v), bromophenol blue (0.002 % w/v), and β-mercaptoethanol (2 % v/v). After dilution, samples were heated to 95 °C for 5 min with stirring, then centrifuged. Electrophoresis was conducted using a Criterion XT 12 % Bis-Tris polyacrylamide gel (Bio-Rad®), alongside a Criterion XT molecular weight marker.

2.2.3. Molecular weight analysis via size exclusion chromatography (SEC)

Chromatographic analysis was performed using an Acquity Ultra-high-Performance Liquid Chromatography (UPLC) system (Waters) with a bioZenTM 1.8 μm, 150 × 4.6 mm column and a bioZenTM SEC-2 precolumn, 4.6 mm (Phenomenex®). A water/acetonitrile/TFA solution (55/45/0.1, v/v/v) served as the mobile phase at a flow rate of 100 μL/min. Samples from the water-soluble fraction were diluted in the mobile phase to a protein concentration of 1.5 mg/mL, then centrifuged at 12,800g for 5 min at room temperature. The injection volume was 3 μL, with absorbance monitored at 214 nm.

SEC with HPLC was used to determine the molecular weight distribution of the MKARE® hydrolysate. Standards and a SEC 130A° column from Agilent Technology® were employed in a Waters Company HPLC-UV system.

2.3. In vitro model of inflammation on human chondrocytes

2.3.1. Treatment of MKARE®

In the process of extracting ESM-F (MKARE®), two distinct fractions

are identified for analysis: the soluble fraction and the hydrolyzed matrix. To prepare soluble fraction (MKARE®-S), the membrane undergoes a gentle extraction process. The hydrolyzed matrix refers to the structural components of the membrane that are not readily soluble. This fraction is obtained by enzymatically hydrolyzing MKARE® to solubilize the membrane, converting it into low molecular weight peptides, and thereby enhancing its bioavailability.

Preparation of soluble fraction (MKARE®-S/ESM—RT-S): 1 g of ESM was resuspended in 10 mL of milli-Q water and agitated magnetically overnight at 4 °C. The mixture was centrifuged at 4 °C, 5000 rpm, and the supernatant was filtered through a 0.22 μm cellulose acetate filter using a syringe. The filtrate was stored at 4 °C for use in *in vitro* assays.

Preparation of enzymatic digestion of ESM (MKARE®-H/ESM-RT-H): ESM was hydrolyzed at 50 mg/mL with papain at 50 °C overnight, with the addition of 30 mM sodium metabisulfite. The ESM liquid was filtered similarly and stored at 4 °C for use in following assays. The ESM filtrate solution was heated to 100 °C for 10 min to inactivate the enzyme before use in chondrocyte *in vitro* assays.

2.3.2. Chondrocyte culture assay

Our *in vitro* model of inflammation was developed in chondrocytes (P2) inflamed with TNF (25 ng/mL). For this purpose, chondrocytes were seeded on 6-well plates, with 4.8×10^4 cells/well. When 80 % cell confluence was reached, samples were treated as described in Table 1.

After 24 h of incubation, cell samples were collected and using for the following experiments.

2.3.3. Cytotoxicity MTT assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazol bromide] assay (Invitrogen®) was carried out on the inflamed and treated chondrocytes. Cell viability and proliferation can be determined through the MTT assay based on the mitochondrial functionality of the treated cells. Chondrocytes were seeded (1.2×10^4 cells/well) on 48 well plates with the experimental conditions. Analysis was carried out after 24 h of culture. After 24 h, the medium was removed, and the plates rinsed with PBS. Then, plates were incubated for 3 h at 37 °C with a medium containing DMEM without phenol red and 10 % MTT, avoiding exposure to direct light. 100 μL of solubilizing solution of DMSO-Isopropanol (1:1) (Fisher Scientific®) was then added and the plates were incubated for 30 min on a PMS-1000i microplate orbital shaker (Grant Bio™). Optical density in each well was quantified at 570 nm with a multiplate spectrophotometer reader Multiskan GO (Thermo Fisher Scientific®).

2.3.4. Quantitative real-time PCR (qPCR)

RNA was extracted from the cultured cells using the GeneMATRIX universal RNA purification kit by EURx®, following the protocol provided by the supplier. Total RNA was quantified using the QUBIT® RNA assay from Thermo Scientific®. For the synthesis of cDNA, 1000 ng of the extracted RNA was employed, using the high-capacity cDNA reverse transcription kit (Applied Biosystems®). Expression levels of aggrecan ACAN, IL-1α, IL-6, and TNF genes were assessed by qPCR. The primers used are listed in Table 2. Beta-actin (ACT-β) was used as a normalization reference. qPCR assays were conducted with the Power SYBR™ Green PCR Master Mix (2 × concentration) (Applied Biosystems®), in a reaction volume of 20 μl, using the StepOne real-time PCR system (Applied Biosystems®), in accordance with the manufacturer's protocol. Relative expression of the target genes was determined using the $2^{-\Delta\Delta Ct}$ comparative method, with normalization to the housekeeping gene, ACT-β.

2.4. In vivo study of the effects of MKARE®

2.4.1. Study design and patients

This study was designed as a randomized, double-blind, placebo-controlled nutritional intervention trial using an excel functional randomization.

Subjects were recruited through various channel information by e-mail notification or on a presential meeting day. The age range of patients selected was 40–66 years with osteoarticular problems. Due to this, the participants exhibited symptoms such as pain, stiffness, or functional limitations in their daily lives. Participants who reported known allergy to eggs or egg products, were pregnant or breastfeeding were excluded of the study All subjects gave written informed consent before data acquisition. Before enrolling in the study, participants received instructions on the proper consumption of the product and guidance on when and how to use the online questionnaires.

2.4.2. Procedure

The protocol was approved by the Ethical Committee of the University of León (Ref:ULE-063-2023) for compliance with the provisions of Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on Data Protection (GDPR).

All study participants were provided with written instructions and received product capsules by postal mail. Starting on day 1 and continuing over 60 days, subjects ingested one capsule per day containing MKARE®, ESM-RT or placebo. The pre-defined primary efficacy endpoint of the study was the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) with Likert analogue scale from 0 a 10. The WOMAC questionnaire is designed for the assessment of lower extremity pain and function in OA of the knee or hip by assessing severity of pain (5 questions), stiffness (2 questions) and limitation of physical function (17 questions). Subjects reported (0 – 10, 0 = no pain at all, 10 = the most intense pain) through an online version of WOMAC questionnaire on days 0, 7, 30, and 60.

2.5. Statistical analyses

In the *in vitro* assay, each experiment was independently conducted three times. The reported results represent the average ± standard deviation of the three values obtained from these independent studies. ANOVA was used to identify significant differences between groups, and Tukey's or Dunnett's post-hoc analysis were then performed. $p < 0.05$ was used to classify results as statistically significant.

In the *in vivo* study baseline comparison was performed to verify randomization amongst the three groups via ANOVA. The evolution from day 0 to 60 was evaluated using a repeated measures univariate analysis of variance (RM-ANOVA) with the Geisser-Greenhouse correction. In both cases, statistical significance was accepted at a $p < 0.05$ after performing Tukey's post hoc analysis. For the Number Needed to Treat (NNT) evaluation, an absolute improvement of 30 % from baseline was considered as the clinically relevant criteria, with 95 % Confidence Intervals (CI).

Table 1

Summary of different experimental conditions in the *in vitro* assay.

Group	Culture medium	TNF concentration	Treatment concentration
1	Serum-free αMEM (Hyclone®) + 1 % penicillin/ streptomycin	25 ng/mL	–
2	Serum-free αMEM (Hyclone®) + 1 % penicillin/ streptomycin	25 ng/mL	0.5 mg/mL ESM-RT-H
3	Serum-free αMEM (Hyclone®) + 1 % penicillin/ streptomycin	25 ng/mL	0.5 mg/mL MKARE®-H
4	Serum-free αMEM (Hyclone®) + 1 % penicillin/ streptomycin	25 ng/mL	1 mg/mL ESM-RT-S
5	Serum-free αMEM (Hyclone®) + 1 % penicillin/ streptomycin	25 ng/mL	1 mg/mL MKARE®-S

Table 2
Primer sequences and conditions used for qPCR.

Gen	NCBI RefSeq	Forward/Reverse (5′-3′)	Tª melting °C	Product size (bp)
ACT-β	NM_001101.3	CCCTCCATCGTCCACCGCAAATGCTCTGCTGTCACCTTCACCGTTCCAGT	59.758.0	131
ACAN	NM_001369268.1	CTGCCCAACTACCCGGCCATTGCGCCCTGTCAAAGTCGAG	72.171.0	200
IL-6	NM_000600.3	ATAACCACCCCTGACCCAACCATGCTACATTTGCCGAA	74.472.5	169
IL-1a	NM_000575.5	AGAGGAAGAAATCATCAAGTTATACTTTGATTGAGGGCG	57.559.2	139
TNF	NM_000594.3	CCTGAAAACAACCCTCAGACGGCCACATCCTCGGCCAGCTCCACGTCCC	77.979.3	155

GraphPad Software® was used for the statistical analysis.

3. Results and discussion

3.1. Evaluation of ES and ESM quality under different storage conditions

Complete ES were obtained from Arandovo® facilities. Determination of the influence of eggshell storage conditions on the quality of its composition is a key factor (Y. Nys J. Gautron & Hincke, 2001). Samples of both ES and ESM from Arandovo® were analyzed to determine whether storage conditions had some impact on the quality of both the eggshell and the final product (MKARE®).

MKARE® is an ESM powder produced in Arandovo® through an industrial hydromechanical separation process. Upon collection of ES, they are processed on the same day to obtain the ESM. This procedure is implemented at the locations where the ES are generated. This timely processing is crucial to preserve all the proteins from the eggshell membrane, ensuring its maximum biological efficacy (Y. Nys J. Gautron & Hincke, 2001). To confirm this, both qualitative and quantitative tests have been conducted to substantiate this hypothesis.

Table 3 shows the values obtained for the free amino groups in the water-soluble fraction of the different samples of the three ES extractions carried out, as well as the total average. Results showed that the sample stored at room temperature (ES-RT) has a higher content of free amino groups, indicative of a higher degree of protein hydrolysis. On the other hand, as expected (Chi et al., 2022), the fresh sample (ES-F) has the lowest content of free amino groups (lower degree of hydrolysis). Upon comparing the hydrolysis levels of two samples from the same batch, ES-RT and frozen (ES-Fr), a significant increase in the free amino group content was observed in the sample stored at room temperature.

The ES is composed of fibrous proteins such as elastin and collagen, which are crosslinked, making it insoluble. Additionally, there exists a group of soluble proteins, such as ovotransferrin, lysozyme, ovocalyxin-36, and more than 400 types that have been identified with proven functionality (Ahmed et al., 2017, 2019; Mine et al., 2023). Fig. 2A shows an SDS-PAGE electrophoresis profile of the water-soluble protein fractions of the three types of ES samples. As observed, both in the fresh sample and the sample stored under freezing conditions, the water-soluble fraction shows intense bands corresponding to proteins with molecular weights around 75 and 37 kDa. The 75 kDa band is attributed to ovotransferrin (Gautron et al., 2001; Nys et al., 2001), while the 37 kDa band has been previously attributed to ovocalyxin 36 (Cordeiro et al., 2013). Other less intense bands are also observed, corresponding to molecular weights of approximately 150 kDa and in the range of 75–50 kDa. In the case of the sample stored at RT, only very weak bands

corresponding to 75 and 37 kDa are detected, indicating that most of the proteins are degraded after storage under RT conditions.

Water-soluble protein fractions were also analyzed using liquid chromatography by Size Exclusion Chromatography (SEC) to verify the degree of hydrolysis. Fig. 2B presents a chromatographic profile corresponding to the ES-F sample (the sample stored frozen; ES-Fr shows a similar profile). A set of high-intensity peaks corresponding to soluble proteins with a molecular weight greater than 5800 Da is observed. The ES-RT image displays a chromatographic profile of the eggshell sample stored at room temperature. In contrast, this profile exhibits significantly reduced peaks, indicating a higher degradation of soluble proteins due to RT storage. These results suggest extensive degradation of the eggshell proteins when stored at RT. These findings are consistent with those obtained from the OPA assay. The samples undergo significant hydrolysis when stored at RT, considerably reducing the proportion of proteins with molecular weight > 5800 Da and increasing the amount of free amino groups and smaller peptides in the water-soluble fraction. Storage under freezing conditions proves effective in limiting the hydrolysis process considerably. Table 4 shows the values obtained for the free amino groups in the water-soluble fraction of the different samples of two ESM extractions. The table shows that the ESM-RT membrane exhibits a higher reaction with OPA (0.6277 mmol), while fresh MKARE® shows a lower reaction (0.3706 mmol). This indicates that more protein degradation has occurred in ESM-RT (breakdown of proteins in ESM-RT), resulting in a higher content of free amino groups that reacted with OPA. This is caused by the storage of ES at RT, which promotes the growth of microorganisms that degrade the proteins (Han et al., 2023a). There are other factors that affect protein stability such as temperature (Lien et al., 2022), but we noticed that storing the ES at RT directly impacted the appearance of the membrane ESM (color change, from white-pink to brown). Therefore, we decided to investigate what was happening and how this change could be affecting the effectiveness of the ESM. It is the first time that it has been demonstrated that it is important not to store the ES at RT to produce ESM powder for consumption as a nutritional supplement, since degrading proteins like ovotransferrin with anti-inflammatory effects which reduces its effectiveness.

SDS-PAGE and SEC profiles of the soluble proteins extracted from MKARE® and ESM-RT membranes are presented in Fig. 2. SDS-PAGE assay shows (Fig. 2A) the existence of a band corresponding to approximately 10–15 kDa in MKARE® that does not appear in the ESM-RT. This band has been previously detected in the protein fraction of the ES and corresponds to lysozyme (Ahlborn et al., 2006; Kaweewong et al., 2013). A large portion of the proteins present in the products could not be identified due to the low solubility of both products in the treatment buffer used. The absence of a clearly detected lysozyme band in ESM-RT product suggest a potential lower concentration or loss of this protein in that product. The SEC results show that the peak intensity of MKARE® is three times higher than that of ESM-RT (Fig. 2B). This finding aligns with expectations, as it was previously confirmed that the soluble proteins from the ES stored at RT were largely degraded, whereas the soluble proteins from a fresh shell, like those used for MKARE® extraction, retained all their soluble proteins intact. This has significant implications for the effectiveness of the ESM as a supplement for osteoarticular issues. More than 400 types of proteins, such as ovotransferrin,

Table 3
Free amino groups present in the water-soluble fraction of the ES samples, determined by the OPA method. Average from three replicates. Statistically significant differences between samples (P < 0.05).

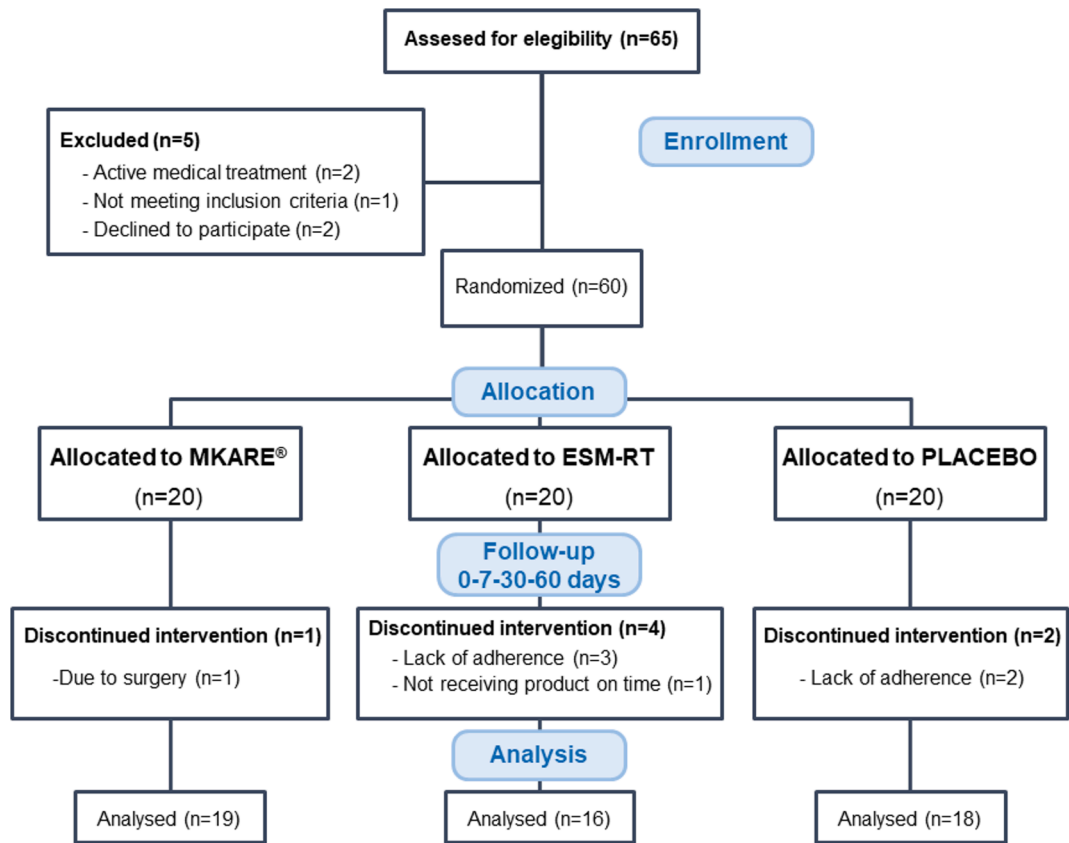|	mmol eq. glutamic acid/kg sample			
	Extraction 1	Extraction 2	Extraction 3	Average
ES-F	2.649	2.760	2.776	2.728 ± 0.069
ES-Fr	3.543	3.990	4.359	3.964 ± 0.409
ES-RT	6.848	8.098	7.978	7.642 ± 0.690

Fig. 1. Enrolment, randomization, and completion flow diagram on the ITT population.
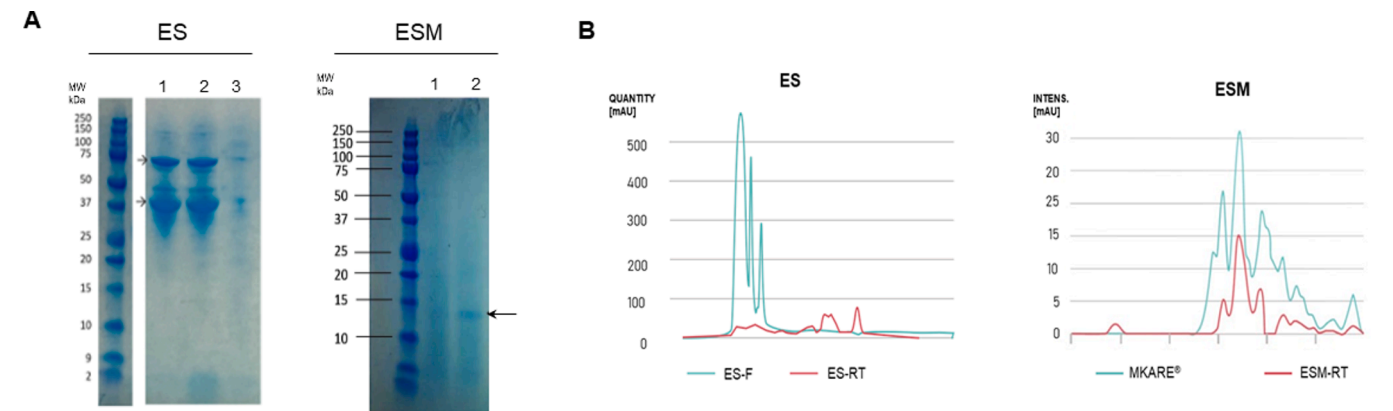

Fig. 2. (A) SDS-PAGE electrophoresis gel under denaturing conditions of samples of ES (1: ES-F; 2: ES-Fr and 3: ES-RT) and ESM (1: MKARE® and 2: ESM-RT). **(B)** Chromatograms obtained by Size Exclusion Chromatography (SEC) of the water-soluble fraction obtained from the different samples of ES (ES-F and ES-RT) and ESM (MKARE® and ESM-RT).

Table 4
Free amino groups present in the water-soluble fraction of the ESM samples, determined by the OPA method. Average from three replicates. Statistically significant differences between samples ($P < 0.05$).

	mmol eq. glutamic acid/kg sample			
	Extraction 1	Extraction 2	Extraction 3	Average
MKARE®	0.3730	0.3632	0.3755	0.3706 ± 0.0065
ESM-RT	0.5773	0.6477	0.6580	0.6277 ± 0.0439

ovocalyxin-36, ovocleidins 17 and 116, lysozyme, etc., have been detected in the ESM. Most of these have demonstrated mineralization and anti-inflammatory effects (Carrillo et al., 2016; Cordeiro et al., 2013; Wu & Acero-Lopez, 2012). If a fresh membrane such as MKARE® retains these soluble proteins, it will offer a bioactivity that a membrane obtained from an eggshell stored at RT cannot provide.

In the realm of ESM research, the freshness of the ES plays a pivotal role in harnessing its maximum potential. This is especially critical when considering the therapeutic proteins present in the eggshell membrane, which are known for their mineralization and anti-inflammatory properties (Matsuoka et al., 2019; Qosimah et al., 2016). To preserve these valuable proteins, it is imperative to take measures to prevent their degradation. When ES are produced and subsequently stored at RT, they

undergo a process of protein degradation and microbial growth, which can lead to a significant loss of bio-functional proteins. This degradation not only diminishes the therapeutic efficacy of the ESM but also affects the overall quality of the extracted material (Rath et al., 2017). Therefore, it is essential to process these eggshells promptly after they are generated to obtain a high-quality, bio-functional product such as MKARE®.

Our experiments reveal distinct differences between MKARE® extracted from fresh ES and those from ES stored at RT for extended periods. The latter showed a marked decrease in the integrity and concentration of bio-functional proteins, underlining the necessity of immediate processing post-generation for optimal results.

3.2. MKARE® have no cytotoxic effects on human chondrocytes in vitro

To carry out the *in vitro* assay, two distinct fractions of ESM were selected for analysis: the soluble fraction (MKARE®-S) and the hydrolyzed matrix (MKARE®- RT- H). The soluble fraction encompasses the readily dissolvable components of the membrane, which may include various proteins, GAGs, and other bioactive molecules. The characterization of this fraction provides insights into the naturally occurring compounds within the membrane that might contribute to its bioactivity. To obtain this soluble fraction (MKARE®-S and ESM-RT-S), the membrane undergoes a gentle extraction process, typically using a buffered solution, to ensure the preservation of its bioactive components (Ahmed et al., 2017). Conversely, the hydrolyzed matrix (MKARE®-H and ESM-RT-H) refers to all components of ESM, the soluble components and the structural components of the membrane that are not readily soluble (fibrous proteins: elastin, collagens…). This fraction is subjected to enzymatic hydrolysis, a process that breaks down the more complex, insoluble proteins and fibers into smaller peptides. This hydrolysis is crucial for revealing the full spectrum of bioactive substances within the membrane, some of which may only be liberated through this process. The methods of extraction and hydrolysis were meticulously optimized to maximize yield and maintain the integrity of the bioactive compounds, ensuring the reliability and relevance of our findings. The profile of molecular weight of the dissolutions of ESM employed in the *in vitro* assay was characterized by SEC and showed a maximum molecular weight of 2944 Da (results not shown). Both fractions, the soluble and the hydrolyzed matrix, offer distinct and complementary insights into the eggshell membrane's biochemical profile (Wedekind et al., 2017). Analyzing these two fractions separately allows for a more comprehensive understanding of the membrane's potential health benefits, including its application in areas such as osteoarthritis treatment, skin care, and nutrition.

To ensure that the ESM had no adverse effects on cell viability, an MTT assay was conducted. In this assay MKARE®-H, MKARE®-S, ESM-RT-H and ESM-RT-S were tested to confirm the compatibility and safety of the ESM in a biological context. In Fig. 3A the proliferation of chondrocytes cultured with the different ESM samples is shown. Cells were viable when they were cultured with all the products according to the results obtained from the MTT assay, though the highest proliferation rate was obtained with both soluble fractions. No significant differences in viability were shown respect to control sample.
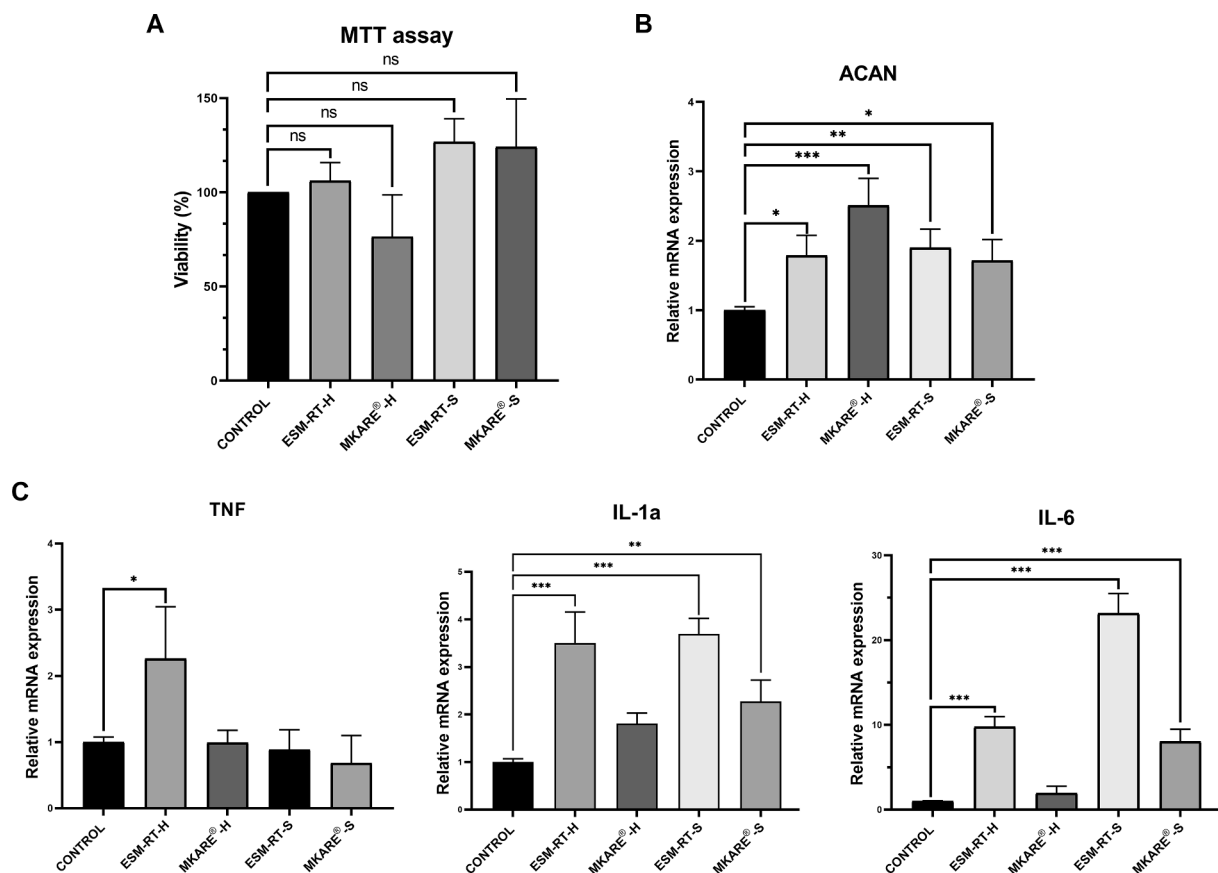

Fig. 3. A) MTT assay of human chondrocyte cells supplemented with ESM. The number of viable cells is expressed as the increased absorbance of the MTT test on 24 h of culture. ns p value > 0.05. **B)** Relative gene expression of ACAN as specific gene of chondrogenesis in human chondrocytes compared for TNF-inflamed chondrocytes, MKARE®-treated TNF-inflamed chondrocytes and ESM-RT-treated TNF-inflamed chondrocytes after 24 h of incubation. **C)** Relative gene expression of specific genes of inflammation TNF, IL-1α and IL-6 in human chondrocytes. MKARE®-treated TNF-inflamed chondrocytes and ESM-RT-treated TNF-inflamed chondrocytes after 24 h of incubation. p value: * $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$. Each bar represents the mean ± SEM of at least three experiments performed in triplicates.

3.3. MKARE® possesses protective and anti-inflammatory effects on human chondrocyte cell model of inflammation

Despite recent advancements in using ESM for various biomedical applications, including cultivation of human fibroblasts on ESM for tissue engineering applications (Vuong et al., 2018), there appears to be a notable absence of studies focusing specifically on the use of ESM in *in vitro* chondrocyte models for OA. Researching the effects of ESM on chondrocytes, particularly within the inflammatory environment, could provide invaluable insights into the development of novel therapeutic strategies for this pathology.

Recent studies have revealed that the release of inflammatory molecules, such as pro-inflammatory cytokines, are essential mediators of altered metabolism and accelerated extracellular matrix degradation (Ashruf & Ansari, 2023; Rapp & Zaucke, 2023; Wojdasiewicz et al., 2014). In our *in vitro* model the effect of MKARE®-H, MKARE®-S, ESM-RT-H and ESM-RT-S added to chondrocyte-inflamed cultures was compared. The expression of specific chondrogenic genes such as ACAN was analyzed under the different experimental conditions stimulated with TNF. Fig. 3B shows significant increases in ACAN gene expression with the addition of all ESM samples. The higher rate was obtained with MKARE®-H sample.

Regarding the inflammatory genes, MKARE®-H dramatically reduced the expression of these mediators (IL-1α and IL-6). The same effect was detected in TNF gene expression though the downregulation observed was not significant, even a significant increase was observed when ESM-RT-H was added. These results are in keeping with other works which have also shown increased expression of IL-1α and IL-6 after TNF inflammation (Porée et al., 2008; Yang et al., 2014). IL-1α and multifunctional cytokine IL-6 is hypothesized to play a role in the inflammatory processes associated with OA by inducing collagen degradation that leads to cartilage erosion and joint destruction, as well as by boosting the production of other pro-inflammatory cytokines such MMPs (Scheller et al., 2011).

Our results showed that MKARE® strongly reduced the expression of pro inflammatory genes activity and increased specific genes of cartilage such as ACAN. In both assays, the greatest effect was observed when MKARE®-H was added to the inflamed chondrocyte cultures.

3.4. Placebo-controlled clinical study of MKARE® effects on patients with knee osteoarthritis

To carry out the clinical study, subjects were recruited through various channel such as e-mail notification or in-person meetings. The age range of patients selected was 40–66 years, media of age: 51.4-year-old. 44 % - male and 56 % - female with osteoarthritic problems with symptoms of pain, stiffness or functionality problems in their daily life. Furthermore, applicants were excluded in case they reported known allergy to eggs or egg products, were pregnant or breastfeeding. All subjects gave written informed consent before data acquisition. Before enrolment subjects were instructed how and when to consume the product and how to use the online questionnaires. Throughout the study, support was available through both telephone and mail.

Although there is growing popularity for these products, particularly in treating osteoarthritis, the scientific evidence regarding their effectiveness is mixed. Some studies in rats have shown positive results in reducing inflammatory cytokines and improving joint swelling when administering ESM (Kevin J. Ruff et al., 2018; Sim et al., 2015; Wedekind et al., 2017). However, it is important to note that these results in animals do not always directly translate to humans.

In the context of human studies, the findings are diverse. Certain studies have reported statistically significant improvements in pain relief and stiffness in patients who consumed ESM (K J Ruff et al., 2009), but other studies have had high dropout rates or have not shown significant improvements in joint function or overall joint health (Kiers & Bult, 2021). Moreover, some studies have lacked a placebo control

group, making it difficult to determine the true effectiveness of the supplement (Aguirre, 2016).

The consort flowchart containing the intent-to-treat (ITT) population is summarized in Fig. 1. In our study, of the 65 patients from the original ITT population, five were excluded due to various reasons. Two subjects were actively being treated owing to chronic pain, one suffered from upper rather than lower articular pain and two declined to participate. The 60 patients left were randomly allocated to one of the treatment groups (20 for MKARE®, 20 for ESM-RT and 20 for placebo). In the following weeks, seven patients dropped out from the study. In the MKARE® group one subject had to undergo surgery thus leaving the treatment. In the ESM-RT group three subjects withdrew due to a lack of adherence to the treatment and another one for not receiving the treatment on time to properly follow the trial. Lastly in the placebo group two subjects withdrew, also due to a lack of adherence to the treatment. Thus, the final total population evaluated was 53 (19 for MKARE®, 16 for ESM-RT and 18 for placebo) with an overall drop-out rate of 11.7 %.

Firstly, the WOMAC baseline score was evaluated to verify randomization amongst the three groups via ANOVA, showing no significant differences between groups at $p < 0.05$.

Post-baseline analyses, focused on the variation of the WOMAC scores throughout the study were carried out. The RM-ANOVA analysis of variance showed a significant overall improvement in patients treated with MKARE®, and ESM-RT compared to day 0 in both 30 (MKARE® mean improvement: 45.43 %, p = 0.002**; ESM-RT mean improvement: 37.38 %, p = 0.03*) and 60 days (MKARE® mean improvement: 51.15 %, p = 0.003**; ESM-RT mean improvement: 32.61 %, p = 0.032*) timespans. No significant improvement over time was observed in the placebo group. Fig. 4, shows the individualized analysis for each sub score and treatment group, consolidating a higher significant improvement in the MKARE® compared to ESM-RT group.

The NNT is a common approach in pharmacology to convey the real efficiency of a new treatment. This figure represents the number of patients needed to be treated to prevent one additional bad outcome (e.g. worsening of an illness). NNTs of 5 or below are normally regarded equivalent to an effective treatment to a pain related condition (Wen et al., 2005). In this study, the NNT value was calculated considering an absolute ≥ 30 % individual overall improvement from baseline as the clinically relevant outcome, and the placebo group as the control standard. Based on this criterion, the MKARE® group showed an Absolute Risk Reduction (ARR) of 40.6 % with an NNT value of 3 (95 % CI, 1.4 to 8.9) at 30 days and an ARR of 40.1 % with an NNT value of 3 (95 % CI, 1.4 to 9.1) at 60 days. On the other hand, the ESM-RT group showed an ARR of 28.47 % with an NNT value of 4 (95 % CI; NNH 29 to infinity to NNB 1.7) at 30 days and an ARR of 11.10 % with an NNT value of 9 (95 % CI; NNH 4.5 to infinity to NNB 2.3).

The results obtained in our work are promising, however, addressing the challenges and prospects of using ESM as a natural alternative in medical applications requires a nuanced understanding of both its unique properties and the broader context of natural product utilization in healthcare. One of the primary challenges lies in the characterization and standardization of ESM, as its bioactive components can vary depending on the source and processing methods. This variability can affect reproducibility and efficacy in clinical applications, needing rigorous quality control and standardized extraction protocols to ensure consistent therapeutic outcomes. Furthermore, translating the benefits of ESM into clinically effective treatments requires extensive research to understand its mechanisms of action, optimal dosages, and potential side effects. Prospectively, the future of ESM in medical applications looks promising, especially with advancements in biotechnology and nanotechnology that can enhance its bioavailability and therapeutic potential. Research into novel delivery systems, such as ESM-based hydrogels or nanoparticles, could open new avenues for its application in regenerative medicine, drug delivery, and beyond. Collaborative efforts between researchers, clinicians, and industry stakeholders will be
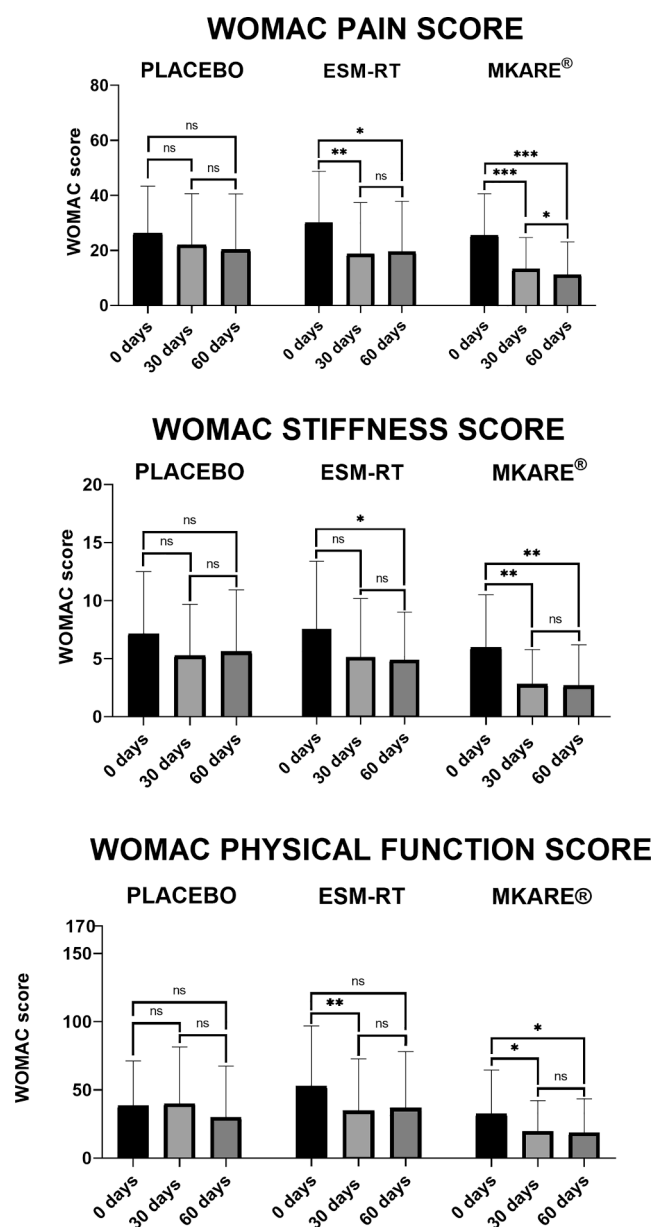
Fig. 4. WOMAC pain score development for each treatment group (subscores: **A)** Pain; **B)** Stiffness; **C)** Physical function). p value: ns > 0.05; * p ≤ 0.05; ** p ≤ 0.01; ***p ≤ 0.001. Each bar represents the mean ± SEM of at least three experiments performed in triplicates.

crucial in overcoming the existing challenges and unlocking the full therapeutic potential of ESM, positioning it as a sustainable and effective natural alternative in the evolving landscape of medical treatments.

4. Conclusion

In conclusion, MKARE® emerges as a promising approach to address inflammation and stimulate chondrocyte regeneration in the context of OA. ESM freshness is a crucial factor; as the membrane ages, it undergoes biochemical changes that can compromise its therapeutic efficacy. The integrity and concentration of vital constituents are best maintained in fresh membranes, making them more effective in promoting joint health and alleviating osteoarticular symptoms. This emphasis on the freshness of the eggshell membrane opens a new perspective in natural osteoarticular therapies, highlighting the potential of utilizing fresh bio-sourced materials for clinical benefits.

Ethics statement

The protocol was approved by the Ethical Committee of the University of León for compliance with the provisions of Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on Data Protection (GDPR).

All study participants were provided with written instructions and received product capsules by postal mail.

Ethics statement

Authors follow a code of ethics.

CRediT authorship contribution statement

Alejandro Casado-Santos: Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manuel A. La Nuez-García:** Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Patricia Álvarez-Rodríguez:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Elsa González-Cubero:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Yaiza González-Rodríguez:** Supervision, Methodology, Formal analysis, Data curation. **Maria Luisa González-Fernández:** Validation, Supervision, Formal analysis, Data curation, Conceptualization. **Vega Villar-Suárez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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