Effects of a low-fat yoghurt supplemented with a rooster comb extract on muscle joint function in adults with mild knee pain: a randomized, double blind, parallel, placebo-controlled, clinical trial of efficacy.

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Running headline of not more than 40 characters (including spaces): yoghurt, rooster comb extract and knee

Abbreviations: ANCOVA, analysis of covariance; **CTNS**, Nutrition and Health Technology Centre; **GCP**, good clinical practice; **HA**, hyaluronic acid; **ICC**, intra-class correlation coefficients; **ICH**, International Conference of Harmonization; **ITT**, intention to treat; **JKOM**, Japanese Knee Osteoarthritis Measure; **KOA**, Knee osteoarthritis; **OA**, osteoarthritis; **PET**, polyethylene terephthalate; **PGE2**, prostaglandin E2; **RCE**, rooster comb extract; **SOCS3**, cytokine signaling 3; **TLR4**, Toll-like receptor 4; **VAS**, visual analogic scale.

Authors' contributions to manuscript were as following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data: RS, R-MV, IM, AR, FP, LA, CC, DM-P were responsible for the overall study design including project concept, development of the research plan, and study oversight.
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AR, CC, DM-P provided essential reagents or materials (applies to authors who contributed by providing animals, constructs, databases, etc. necessary for the research)
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ABSTRACT

Preliminary results suggested that oral-administration of rooster comb extract (RCE) rich in hyaluronic acid (HA) was associated with improved muscle strength. Following these promising results, the objective of the present study was to evaluate low-fat yoghurt supplemented with RCE rich in HA on muscle function in adults with mild knee pain; a symptom of early osteoarthritis. Participants (n=40) received low-fat yoghurt (125 mL/d) supplemented with 80 mg/d of RCE and placebo group (n=40) consumed the same yoghurt without the RCE, in a randomized, controlled, double-blind, parallel trial over 12 weeks. Using an isokinetic dynamometer (Biodex System 4), RCE consumption, compared to control, increased affected knee peak torque, total work and mean power at 180º/s, at least 11% in men ($p<0.05$) with no differences in women. No dietary differences were noted. These results suggest that long-term consumption of low-fat yoghurt supplemented with RCE could be a dietary tool to improve muscle strength in men, with attendant possible clinical significance. However, further studies are needed to elucidate reasons for these sex difference response observed, and may provide further insight into muscle function.

Trial Registration: ClinicalTrials.gov Identifier: NCT01303432.

Word count = 180

Key words: knee discomfort, rooster comb extract, novel food, hyaluronic acid, muscle strength, isokinetic dynamometer

1. INTRODUCTION

Knee osteoarthritis (KOA) is a major public health problem [1] because it causes chronic disability in older people [2]. Early KOA is recognized by knee discomfort with no clear lesions or associated abnormalities, and requires a conservative approach in first choice management [3]. Muscular functional limitations have been targeted in developing tools directed at underlying mechanisms for KOA [4]. The muscles around the knee, particularly the quadriceps and hamstring, are known to act as dynamic stabilizers. The knee extensors are the main movers involved in physical activities such as running, jumping or kicking the ball, whereas the knee flexors are involved in physical activities where they influence stride length and stabilize the knee joint in changes of direction, acceleration and deceleration, and during landing [5,6]. Both, knee extensor and knee flexor strengths are lost with the progress of symptomatic KOA [7]. Weakness of knee flexor and extensor muscles could lead to a decreased joint stability which, combined with the decreased biomechanical efficiency, leads to debilitating falls, especially in the elderly [7–9]. The quadriceps muscle weakness may precede KOA [8,9].

Isokinetic muscle strength, which is evaluated by peak torque, total work and mean power, can identify muscle weakness and can assist the diagnostic process, or can be used to determine the effect of following different interventions [10,11]. Strength of the knee flexors and extensors has been identified as an important parameter in prevention of injury of knees [12] . Depending on the joint, the muscle group and the movement to be studied, multiple angular velocities were used. To evaluate muscle function, the measurements included slow velocities (up to 60 °/s), intermediate velocities (90 to 120 °/s) and fast velocities (180-300º/s). The maximum work is lower at higher movement velocity. Of note is that velocity of 180º/s appears to have gained general acceptance and, currently, is being used widely [13,14].

Hyaluronic acid (HA) is composed of N-acetyl glucosamine (as the monosaccharide) together with D-glucuronic acid. Glucosamine can prevent cytokine-induced DNA demethylation of a specific CpG site in the IL1β promoter resulting in a decrease of expression via NF-kB in human chondrocytes [15,16]. In addition, HA has a key role in myogenesis and regulation of myocites cycle [17]. Further, orally-administered HA is absorbed and ubiquitously distributed in organs and joints [18], thus opening possibilities for developing therapies to treat discomfort in various joints. A study with horses with osteochondrosis demonstrated that the oral-administration of rooster comb extract (RCE) rich in HA for a period of 90 days increased the intra-articular concentration of hyaluronic acid [19]. A preliminary study [11] involving intake of a low-fat yoghurt containing added RCE produced a significant increase in the maximum peak torque of the knee extensors at 180º/s and at 240º/s, while a similar pattern of response was observed in total work and in mean power; the outcome being improved muscle strength and flexibility [11]. These promising results offer new therapeutic opportunities, albeit studies with higher levels of scientific evidence are needed. RCE underwent a safety assessment and, as a result of which, an authorization decision was taken by the European Commission based upon a positive assessment by European Food Safety Authority [20].

Foods naturally containing sodium hyaluronate are very limited. Only viscera and rooster combs have high amounts of this substance. Cultural habits (not all countries include rooster combs and/or viscera in their diets) often precludes these products in a regular diet. Hence, a good way to make up this lack in sodium hyaluronate intake could be to include rooster combs extract (RCE) in foods which are daily consumed, such as dairy products.

Our hypothesis is that the consumption of a low-fat yogurt supplemented with RCE improves muscle strength of the quadriceps and hamstring muscles in patients affected with mild knee pain, resulting in greater knee joint stability.

The objective of this study is to determine the effect of intake of low-fat yoghurt supplemented with RCE on muscle strength of the affected knee joint, as determined by an isokinetic gold standard method. Additional evaluations included an echography, subjective assessment of pain, and safety of the RCE-supplemented low-fat yoghurt.

2. MATERIALS AND METHODS

2.1 Study design, randomization and intervention

The study was a randomized, double-blind, placebo-controlled, two-arm study assessing the effect of RCE on joint function in adults with mild knee pain. The randomization code was computer generated. The randomization list was based on a block randomization procedure (with block-size of 4) generated using PROC PLAN in the SAS program (version 9.2). To guarantee allocation concealment, the randomization list was guarded and was unavailable to investigators involved in the study. Participant assignment to treatment or placebo arm was at a ratio of 1:1. The number sequence for the subject, center, and treatment assignment were allocated via an interactive electronic response system hosted by the Nutrition and Health Technology Centre (CTNS). The Unit responsible for the randomization took no further part in the study.

Participants were randomized to receive a low-fat yoghurt (125 mL/d) supplemented with 80 mg/d of RCE (Mobilee®; Bioiberica S.A., Palafolls, Spain), or the same low-fat yoghurt without RCE, over a period of 12 weeks. The dose and treatment duration followed that of previous studies [21,22]. The RCE was extracted from food grade rooster combs using an extraction process. To guarantee the appropriate dosage, the RCE was added before yogurt fermentation in the manufacture process. The concentrations,

147 structure and stability of the RCE were confirmed before the yogurts were made
148 available to the participants. RCE contained HA (65%) together with hydrolyzed
149 proteins (particularly collagen) and other polysaccharides. The content of HA in the
150 final yogurt product was determined according to the method described by Coleman et
151 al 1997 [23]. Each 100g of the low-fat yoghurt contains: 3.25% protein; 0.2% fat; 4.45%
152 carbohydrates; 30 kcal. The only difference between investigation product and control
153 was the supplementation with RCE (80 mg/unit). The participants were asked to
154 consume the yoghurt at the same time each day; preferably at lunchtime.
155 The participants' diets were monitored using two 3-day dietary records, one prior to
156 commencing the study, and the other at 12 weeks of the trial. Additionally, a list of
157 foods and products rich in mucopolysaccharides and/or HA was provided to participants
158 with instructions to avoid these dietary items so as to preempt their influence on the test
159 substance measurements.

160 **2.2 Participants**

161 Participants were outpatients at the *Hospital Universitari Sant Joan* (Reus, Spain). All
162 had been suffering from mild pain knee (evaluated on VAS as being between 30 mm
163 and 50 mm), for a minimum of 6 months. The exclusion criteria were: 1) regular use of
164 paracetamol or other drugs to control joint discomfort; 2) active rheumatoid arthritis and
165 any inflammatory arthritic conditions 3) treatment with oral corticosteroids within the 4
166 weeks prior to selection; 4) treatment with intra-articular corticosteroids within the 12
167 weeks prior to selection; 5) significant joint injury during the 3 weeks prior to
168 screening (identified from medical history); 6) patients who consume drugs or dietary
169 supplements for osteoarthritis (OA) at the time of screening; 7) individuals who depend
170 on prescription drugs to control pain; 8) patients participating in a concurrent clinical
171 trial, or having received a product being evaluated during the previous 30 days; 9)

allergy to dairy products; 10) individuals following an energy-restricted diet for weight loss; 11) pregnant or lactating; 12) currently taking nutraceuticals with HA and/or other types of joint regenerators; 13) suffering from axis alterations. Baseline characteristics of the participants are summarized in Table 1. Participant flow throughout the study is shown in Figure 1.

The study was conducted between February 2011 and June 2011 in *Hospital Universitari Sant Joan* (Reus, Spain). The adverse events were coded according to the Medical Dictionary for Regulatory Activities (MedDra dictionary; version 9). We approached the present manuscript when RCE had been approved by the European Commission as a Novel Food ingredient [20].

2.3 Packaging characteristics

The investigational and control products were packed in 125 mL polyethylene terephthalate (PET) containers sealed with an aluminum foil cover. The test units were batched in cartons containing 6 units each. The labels on each included the following information: EU code for products of animal origin, consume-by date, trial code / name of the promoter, the inscription "sample for nutritional investigation", storage conditions, blank space for noting information, consume-by date if necessary, and participant's code identification number in the study. The palatability and general acceptability of the low-fat yoghurt supplemented with RCE compared with the placebo was evaluated by means of a subjective acceptance questionnaire.

2.4 Ethics

The study was approved by the Clinical Research Ethical Committee of the *Hospital Universitari Sant Joan.* Protocol was according to the Helsinki Declaration and good clinical practice guidelines of the International Conference of Harmonization (ICH

GCP). All participants provided written informed consent prior to enrolment into the trial.

This trial was registered with ClinicalTrials.gov: number NCT01303432.

2.5 Outcomes

Main outcome: To assess evolution of muscle function over 12 weeks from baseline as measured by isokinetic evaluation of the affected knee joint

Secondary outcomes: To assess change over 12 weeks from baseline in the echographic evaluation of the affected joint using an OA risk parameter scale, and pain evaluation on the VAS scale

2.6 Clinical assessment

Isokinetic test: The evaluation was conducted with an isokinetic dynamometer as gold standard method (Biodex System 4; Biodex Medical Systems, New York, USA) using five repetitions at two angular velocities (180º/s, 240º/s) [24]. This allows a quantitative evaluation of muscle function through variables such as torque, work and power. As we have observed, the maximum work is lower at higher movement velocity, thus fast velocities such as 180º/s and 240º/s would be optimal for our purpose. The participant assumed a seated position with the hips flexed at 90º. The degree of freedom of the knee was restricted to extension/flexion of 0 to –90. A break of 2 min was allowed between sets of measurements. Based on the data retrieved from all the sets, the maximum total work (J), maximum peak torque (Nm) and mean power (W) at 180 and 240º/s were determined. The maximum peak torque (Nm) was defined as the maximum force produced by the tested musculature at the two different angular velocities. Total work (J) was defined as the workload at a defined angular velocity, while mean power (W) was defined as total work over a specific period of time [11].

The intra-and inter observer reliability (consistency) of the isokinetic strength-testing protocol for knee extension and flexion was determined[25]. For inter-observer, the intra-class correlation coefficients (ICC) of the isokinetic variable peak torque was 0.91 (95%CI: 0.85-0.97) and for intra-observer, the ICC was 0.95 (95%CI: 0.70-0.99), both representing 'good' to 'very good' reliability according to Landis and Koch interpretation [26].

2.7 Statistical analyses

Sample size was calculated using the results obtained in a previous trial [15] on the isokinetic evaluation of peak torque under specific analytical conditions of 240º in extension. Assuming a standard deviation (SD) of 8.5 Newton (Nm), 40 participants per group were necessary to detect differences between the two groups (placebo and experimental) of 5.5 Nm under an α=0.05 significance level, and a power of 80%. Descriptive results were expressed as mean±standard deviation (SD) or percentages, according to the variable being measured.

To compare the effects of the two products (test and placebo) on the efficacy of the principal variable, as well as on the main secondary efficacy variables, an analysis of covariance was performed (ANCOVA) with the baseline value as covariate. The studied population was analyzed by intention-to-treat, defined as all randomized subjects who met inclusion/exclusion criteria, who received the study products (placebo or active-ingredient yoghurts), and had at least a baseline efficacy measurement. For the main efficacy analysis, missing values were imputed by means of the Baseline Observation Carried Forward (BOCF) method, and sensitivity analysis based on Available Data Only (ADO) approach were also performed, finding no remarkable differences, improving the robustness of the statistical results. For the rest of efficacy variables,

hypotheses were tested using Fisher's exact test for categorical variables, the Student's *t*-test for continuous variables and Mann-Whitney's U-test for ordinal variables.

All statistical analyses were performed with the SAS 9.2 (SAS Institute, Cary NC) package. Significance level was fixed at bilateral 5%. Previous to the opening of the randomization codes and the lock of the database, a Statistical Analysis Plan was performed, and all analyses were conducted in accordance.

3. RESULTS

3.1 Baseline characteristics of the study participants

From the 89 eligible volunteers, 84 were randomized and 80 were analyzed (30 men and 50 women). The mean (±SD) age was 42.52±13.16 years and the BMI was 25.36±3.72 kg/m2, as described in Table 1 and Figure 1.

3.2 Attrition rates

At 12 weeks, both groups had 95% adherence to the study protocol and no statistically significant differences in attrition rates were observed between intervention and control groups (P = 0.89).

3.3 Evaluation of compliance, tolerance with the product and adverse events

Of the participants, 76 (94%) included in the safety population completed the trial without significant protocol deviations; 93% (n=37) in the placebo group and 95% (n=39) in the intervention group. The palatability and general acceptability of the low-fat yoghurt supplemented with RCE was well and no differences were observed with placebo. Adverse events were reported in 9 volunteers and were related to gastrointestinal discomfort such as flatulence and stomach ache. The severity of the adverse events was mild and in none of the cases the intervention was modified or interrupted. Moreover, there were no statistically significant differences between groups with respect to adverse events reported.

3.4 Dietary intake

The intake of energy, macronutrients, cholesterol and alcohol did not change during the 12 weeks intervention period, and no significant differences were observed between groups.

3.5 Isokinetic evaluation of muscle function in the affected knee joint

No significant differences were observed when comparing RCE group isokinetic variables with placebo globally (Supplementary Table 1). When the isokinetic data on peak torque (Nm), total work (J) and mean power (W) at 180º/s and 240º/s is segregated by gender, significant differences where observed in men. At 12 weeks, men in the RCE group significantly increased the muscle strength in the affected knee-joint in flexion and extension improving the mainly isokinetic variables measured at 180º/s and also at 240º/s compared to placebo. The % of change from baseline in the RCE intervention is in all the isokinetic parameters over 19%. Moreover, the % of difference from placebo is in all the variables determined over 11%. No statistically significant changes were observed in women between RCE and placebo. The most relevant isokinetic data at 180º/s and segregated by gender are summarized in Table 2.

3.6 Effusion of affected knee joint

The effusion of the affected knee joint was evaluated using echography, and no significant changes were observed between control and intervention groups. At 12 weeks, the RCE-supplemented group had a reduction of -5.35 % (in mm) while, in placebo group, this was increased by +1.92 %; albeit the difference was not statistically significant (P=0.276).

3.7 Pain evaluation

Pain evaluation on the VAS scale showed no statistically significant differences between intervention and control group.

4. DISCUSSION

The present study confirms that low-fat yoghurt supplemented with a natural compound RCE rich in HA (80 mg/d) consumed over 12 weeks can improve the muscular status in the affected knee-joint, at least a 11% in men, compared to control group. Peak torque, total work, and mean power in flexion and extension evaluated in two angles (180º/s and 240º/s) increased at least 19% in men suffering from mild knee pain compared to baseline. From our knowledge, the information about clinical significance of knee isokinetic measures improvement is scarce. It is proposed that when the comparison between two isokinetic variable data is greater than 10% is generally considered as being functionally significant [27,28]. Thus, the improvement in the affected knee-joint muscle strength that was observed after the RCE intervention could suggest a clinical practical importance leading to clinical significance [29].

In a healthy population, women have lower muscle strength than men at all age groups. Male muscle strength declines progressively and linearly with age, while female muscle strength decreases from around the age of 41 years [10]. Kasai et al.[30] observed sex and age related differences in thigh cross-section area, composition and muscle quality. With age the thigh cross-sectional area decreases mainly because of a reduction in muscle in men and, in contrast, because of fat reduction in women. Moreover, the rate of decrease in muscle cross-sectional area was 1.5-fold higher in men than in women. However, different studies have suggested that loss of ovarian function associated with decreased circulating concentrations of 17b-estradiol could indirectly be associated with the accelerated decline in muscle strength after the menopause [31]. Hence, sex steroidogenesis-related mRNA and protein expressions, such as for 17β-hydroxysteroid dehydrogenase (HSD), 3β-HSD, 5α-reductase and aromatase cytochrome P-450 (P450arom) enzymes, are detected in the skeletal muscle while testosterone, estradiol,

and 5α-dihydrotestosterone are locally synthesized in skeletal muscle from dehydroepiandrosterone [32] . Therefore, acute exercise may increase muscle estrogen synthesis in males, and may increase testosterone synthesis in females. Indeed, muscle estrogen levels were observed to be increased in males, while muscle testosterone levels in females were increased by acute exercise [32]. This interesting approach to muscle metabolism suggests that the difference in sex steroidogenesis enzymes and sex steroid hormone levels in skeletal muscle could be upregulated by products as RCE, and the response may be higher in men. However, future studies are needed to elucidate reasons for these sex difference response and may also provide further insight into muscle function. In the present study, muscle measurements were performed using gold standard methodology with a dynamometer and a computerized system that enables arcs of movement to be measured at a constant angular velocity [33]; the objective standardized isokinetic assessment being the most accurate method to evaluate muscle activity [33]. In KOA, the loss in extensor and flexor strength is attributed to weakness of the quadriceps muscle because its strength (peak torque generation) is an important determinant of physical function in subjects with KOA [7]. Muscle impairments in patients with KOA are not limited to quadriceps, but also involve hamstring muscles [7]. In individuals with KOA, a decrease in the external flexion moment has been reported, and is believed to be a compensation strategy employed to reduce load on the knee joint [34]. The present results suggest that RCE consumption can improve impairments in affected knee muscle strength.

The differences in muscle activity following RCE consumption were not translated into changes in pain perception, probably due to the low intensity of the baseline pain. The muscle activity could be related to intrinsic hyaluronan synthesis, which is necessary for myoblasts to differentiate and form syncytial muscle cells [17]. Similarly, RCE has been

shown *in vitro* in human synovial fibroblasts to have a concentration-dependent effect consistent with the stimulation of endogenous HA synthesis [35]. Since endogenous synthesis of hyaluronan is associated with myogenesis, the effects of RCE consumption on muscle function could be explained by an improvement in myogenesis, which would widen the current perspectives on OA prevention.

The efficacy of the oral administration of HA had been observed [36] in sixty individuals with OA (Kellgren-Lawrence grade 2 or 3) who were randomly assigned to HA (200 mg once a day) or placebo for 12 months. The subjects in both groups were required to perform quadriceps strengthening exercises every day, as part of the treatment. The improvement tended to be clearer with the HA group, and this trend was more obvious with the subjects aged <70 years. For the relatively younger subjects, the oral HA effect was better than in the placebo group at the 2nd and 4th months after the start of consumption [36].

The clinical and biochemical effects of 250 mg/d oral RCE (65% HA) were measured in young horses with osteochondrosis at time 0, at the end of treatment (90 d) and thereafter (every 30 d). The results indicated that animals receiving the RCE supplement had a lower score for synovial effusion as well as higher HA, nitric oxide and prostaglandin E2 (PGE2) concentrations in synovial fluid; the differences, however, did not reach statistical significance compared to control [19] .

The effusion values observed in our volunteers were between 10 and 11 mm which indicate suspicion of pathological joint effusion [37,38]. Although no statistically significant differences were observed between groups, an effusion reduction tendency of – 5.35% (in mm) was shown after RCE intervention indicating that RCE could also had a beneficial effect on this parameter.

Orally administered HA is absorbed and ubiquitously distributed to joints. Experimental results in rats and beagles using radiolabelled HA indicated that orally administered HA would be absorbed and distributed to the skin, bone, and synovial joints, including knee joints, and would be retained in these tissues for protracted periods. The pattern of distribution within the body and the time-course of clearance from the tissues indicated that a substantial part of orally administered HA would be absorbed, without substantial degradation [18]. The oral absorption as well as distribution and excretion of hyaluronic acid (HA) have been studied [39,40] . The percentage of the ingested dose of HA entering systemic circulation is similar to that reported for other glycosaminoglycans (between 5-20%) [39]. Oral absorption of RCE has been determined using the ex vivo everted gut sac model in rats [40]. Intestinal absorption was confirmed using this model, with absorption rates estimated to range between 38% in duodenum and 9% in ileum. That HA reaches peripheral tissues, especially joints and skin, has also been demonstrated [18]. The uptake and transport of high-molecular weight glycosaminoglycans has been suggested to occur through the lymphatic system[18,41].

However, therapeutic effects of HA on KOA patients may not necessarily require the absorption of HA. A recent study by Asari et al [42] reported that a high molecular weight HA can bind to Toll-like receptor 4 (TLR4) at intestinal epithelium, and exert biological activity without being absorbed; the association of HA with TLR4 was shown to increase the secretion of suppressor of cytokine signaling 3 (SOCS3), which leads to the suppression of pro-inflammatory cytokine expression. The binding of HA to TLR4 also suppresses the expression of pleiotrophin which, again, contributes to the suppression of inflammation. Thus, the therapeutic effects of HA observed in the study [42] may have resulted from these mechanisms, with the HA remaining in the intestines without absorption.

Another possibility is that the therapeutic effect of HA is obtained via mechanisms similar to glucosamine. Glucosamine is another supplement which can alleviate symptoms of KOA, and can inhibit the progression of the disease. Although its mechanism is not fully understood, glucosamine is thought to inhibit disease progression by exhibiting chondro-protective and anti-inflammatory activities [15]. N-acetyl glucosamine is the monosaccharide that forms HA in combination with D-glucuronic acid. Recently, the potential for glucosamine to modulate NF-kB activity and cytokine-induced abnormal gene expression in human articular chondrocytes isolated from the articular cartilage of femoral heads following fractured neck-of-femur surgery, was proposed to occur via an epigenetic process. Glucosamine can prevent cytokine-induced demethylation of a specific CpG site in the IL1β promoter, and this can decrease the expression of IL1β. These studies provide a potential mechanism-of-action for KOA disease-modifying agents via NF-kB. These findings demonstrate the need for further studies to elucidate the role of NF-kB in DNA demethylation in human chondrocytes [16]. It is possible that N-acetyl glucosamine released from orally-ingested HA may improve KOA symptoms in the same manner as glucosamine [35].

The strength of the present study is its design as a randomized, controlled, clinical trial which provides first level of scientific evidence. One potential limitation of the study is that it is underpowered to detect differences in sub-group analysis based on sex and, therefore, the sub-analyses conducted need to be considered exploratory. Another limitation is that the mechanisms-of-action of oral RCE consumption were not assessed. Further research is needed to confirm the results described, and to define the mechanisms-of-action of oral RCE. Confirmation of these findings in other groups of patients with mild knee pain of muscular origin could be socio-economically valuable.

5. CONCLUSIONS

In conclusion, long-term intake of low-fat yoghurt supplemented with HA-containing RCE increases muscle strength in men with possible clinical significance, including better performance of the quadriceps and hamstring muscles of the knee. These findings could provide the basis of new dietary therapeutic objectives in the treatment of early osteoarthritis. However, further studies are needed to elucidate reasons for these sex difference response observed, and may provide further insight into muscle function.

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COMPETING INTEREST STATEMENT

Daniel Martínez-Puig and Carlos Chetrit are the only employees of Bioiberica S.A. They participated in the study as experts on the use of the product under investigation. Bioiberica S.A as a corporate entity had no role in data acquisition and the interpretation, or in manuscript preparation and the decision to submit it for publication. The authors also declare there have not been any other involvements such as employment, consultancy and product patents that can be construed as conflicts of interest.

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524 **FIGURE LEGEND**

525 **Figure 1. Flow of participants through the study**

526 Intervention: low-fat dairy product (125 mL/d yoghurt) with 80 mg/d of Rooster Comb

527 Extract (**RCE**) over 12 weeks. Control: the same low-fat yoghurt without RCE over 12

528 weeks. ITT: statistical analyses: intention-to-treat; PP: per protocol.

529

530 **Table 1.** Baseline characteristics of the study participants

Variable	Placebo group n=40	RCE group n=40
Age; years	43.10±13.14	42.38±10.16
Weight; Kg	69.32±13.46	71.55±14.26
Height; cm	166.83±8.50	166.10±11.45
Body Mass Index; Kg/m^2	25.06±3.72	25.64±4.95
Gender; male, n (%)	16 (40.0%)	14 (35.0%)
Race; Caucasian, n (%)	40 (100.0%)	40 (100.0%)
> 50 years of age, n (%)	13 (32.5%)	12 (30.0%)
BMI > 30 Kg/m^2, n (%)	4 (10.0%)	8 (20.0%)

531

532 RCE: low-fat yoghurt supplemented with a rooster comb extract (RCE) rich in

533 hyaluronic acid (65%)

534

535 **Table 2.** Change in the isokinetic values of the affected knee muscle function, segregated by gender

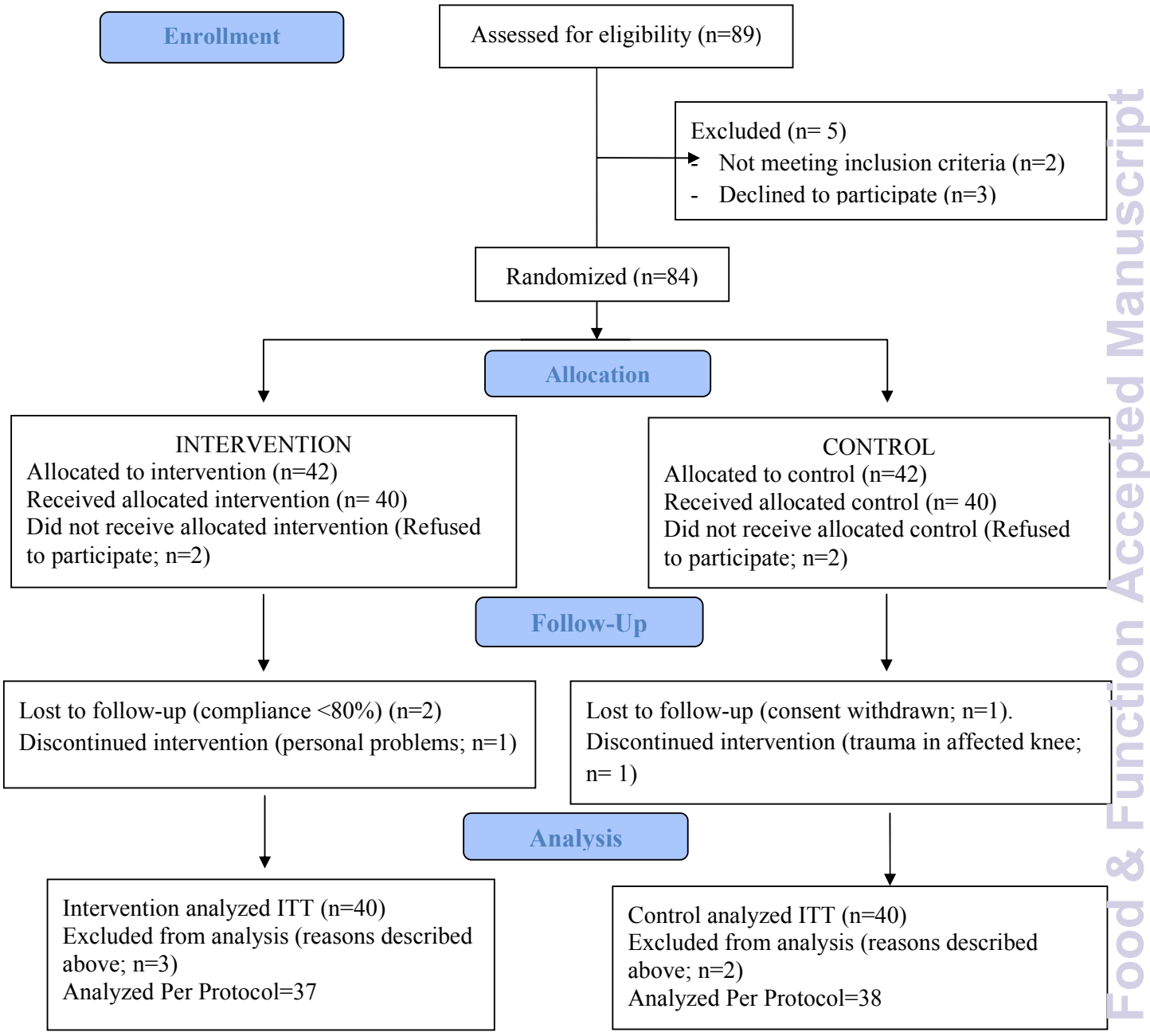
Gender	Parameter	Position	Angle (°/sec)	Treatment	Baseline Mean±SD	Change at 12 weeks relative to baseline Adjusted mean [95%CI] (% change from baseline)	Changes of RCE vs placebo Adjusted mean [95%CI] (% difference from placebo)	P RCE vs. Placebo
Males	**Peak Torque (Nm)**	**Extension**	**180**	**Placebo**	96.23±45.01	7.20 [0.20; 14.20] (7.48%)	16.14 [0.11; 32.17] (11.85%)	0.048
				RCE	112.86±37.28	21.82 [5.64; 38.01] (19.33%)		
		Flexion	**180**	**Placebo**	48.99±25.11	6.24 [1.66; 10.83] (12.74%)	10.21 [2.92; 17.50] (12.67%)	0.007
				RCE	60.89±19.58	15.47 [9.47; 21.47] (25.41%)		
	Total Work (J)	**Extension**	**180**	**Placebo**	476.56±262.99	75.09 [19.86; 130.33] (15.76%)	139.1 [23.00; 255.1] (22.21%)	0.020
				RCE	539.76±195.15	204.94 [86.61; 323.26] (37.97%)		
		Flexion	**180**	**Placebo**	239.47±161.55	60.59 [24.92; 96.25] (25.30%)	74.53 [15.94; 133.1] (17.68%)	0.014
				RCE	294.33±135.03	126.49 [71.36; 181.62] (42.98%)		
	Mean Power (W)	**Extension**	**180**	**Placebo**	140.34±83.24	35.76 [16.43; 55.08] (25.48%)	46.32 [5.00; 87.64] (21.77%)	0.029
				RCE	167.76±67.95	79.26 [38.26; 120.27] (47.25%)		
		Flexion	**180**	**Placebo**	73.04±55.07	22.19 [9.96; 34.42] (30.38%)	25.56 [3.93; 47.19] (18.52%)	0.022
				RCE	92.18±44.52	45.08 [24.70; 65.46] (48.90%)		

Females	**Peak Torque (Nm)**	**Extension**	**180**	**Placebo**	55.10±19.21	10.43 [3.57; 17.30] (18.93%)	-1.37 [-8.84; 6.10] (-1.37%)	0.713
				RCE	55.17±19.54	9.69 [4.60; 14.77] (17.56%)		
		Flexion	**180**	**Placebo**	30.43±12.79	4.33 [-0.76; 9.43] (14.23%)	-0.065 [-5.29; 5.16] (4.27%)	0.980
				RCE	28.11±10.85	5.20 [2.28; 8.13] (18.50%)		
	Total Work (J)	**Extension**	**180**	**Placebo**	278.51±119.02	54.70 [11.31; 98.09] (19.64%)	11.47 [-36.09; 59.03] (9.04%)	0.629
				RCE	260.01±108.65	74.56 [44.42; 104.69] (28.68%)		
		Flexion	**180**	**Placebo**	119.04±75.00	33.54 [4.33; 62.74] (28.18%)	15.03 [-19.26; 49.31] (17.51%)	0.381
				RCE	112.48±69.82	51.39 [28.96; 73.81] (45.69%)		
	Mean Power (W)	**Extension**	**180**	**Placebo**	83.35±38.27	26.77 [12.33; 41.21] (32.12%)	-2.67 [-18.87; 13.54] (2.02%)	0.741
				RCE	77.63±40.95	26.50 [16.14; 36.86] (34.14%)		
		Flexion	**180**	**Placebo**	35.83±23.76	15.24 [5.68; 24.81] (42.53%)	-0.17 [-11.30; 10.96] (4.60%)	0.976
				RCE	33.59±24.24	15.83 [8.86; 22.80] (47.13%)		

All results are expressed as means ± standard deviation and baseline adjusted least square means [95%CI]. Values computed on ITT population by ADO approximation. ANCOVA model

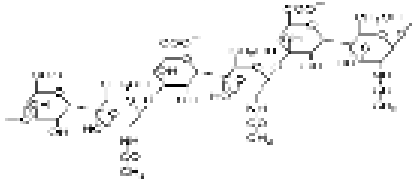
RCE·: a low-fat yoghurt supplemented with a rooster comb extract rich in hyaluronic acid (65%)

Figure 1.

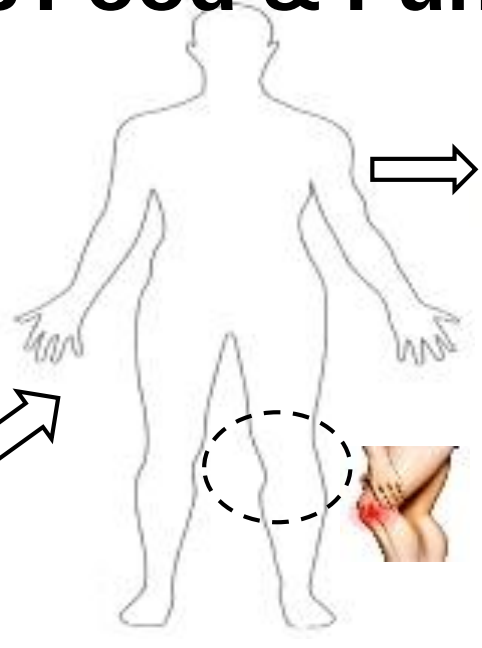


SUPPLEMENTED LOW-FAT YOGHURT

ROOSTER COMB EXTRACT



HYALURONIC ACID



ADULTS WITH MILD KNEE PAIN

ISOKINETIC DYNAMOMETER

- Knee peak torque
- Knee total work
- Knee mean power

AFFECTED MUSCLE STRENGTH