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RESEARCH ARTICLE

Green-lipped mussel extract (*Perna canaliculus*) and glucosamine sulphate in patients with knee osteoarthritis: therapeutic efficacy and effects on gastrointestinal microbiota profiles

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Abstract
Objective To investigate how changes in the gastrointestinal tract (GIT) microbiota profile may influence nutraceutical efficacy in osteoarthritis (OA) and allow the formulation of a hypothesis that explains in part the inconsistent and contentious findings from OA clinical studies with green-lipped mussel (GLM) and glucosamine.
Methods A non-blinded randomised clinical trial was conducted with 38 subjects diagnosed with knee OA. Each participant received either 3,000 mg/day of a whole GLM extract or 3,000 mg/day of glucosamine sulphate (GS), p.o. for 12 weeks. Faecal microbial analyses were carried out after collecting stools at T_0 and T_{12} weeks. Additional pharmacometric measures were obtained from changes in arthritic scores in the Western Ontario McMaster Universities Arthritis Index (WOMAC) and the Lequesne algofunctional indices and the Gastrointestinal Symptom Rating Scale (GSRS). An intention-to-treat analysis was employed and participant data collected at T_0, T_6 and T_{12} weeks.
Results There were no statistically significant changes in bacterial growth patterns determined by the Wilcoxon test. In both groups there was a trend towards a decrease in *Clostridium* and *Staphylococcus* species and increase in *Lactobacillus, Streptococcus* and *Eubacterium* species. In the GLM group *Bifidobacterium* tended to increase and *Enterococcus* and yeast species to decrease. The GS-treated group demonstrated a trend towards a decrease in *Bacteroides* and an increase in yeasts and *Coliforms* species, most notably *Escherichia coli.* We further confirm significant improvement ($p < 0.05$) in all OA outcome measures from T_0 to T_{12} weeks for both the GLM and GS groups. The GSRS scores indicated that GIT function significantly improved over the 12 weeks duration with GLM and GS supplementation.
Conclusion Both GLM and GS reduced OA symptoms and non-significantly altered the gut microbiota profile from baseline. Changes in the microbiota profiles occurred in both treatment groups; the most notable being a reduction in the *Clostridia* sp. This study suggests that nutritional supplements such as GLM and GS may regulate some of the metabolic and immunological activities of the GIT microbiota. The decrease in *Clostridia*, a potent modulator of colonic Th17 and CD4+ regulatory T cells, was consistent with a decrease in inflammation; improved GSRS scores and OA symptoms for these OA participants. The GIT microbiota may be important factor in the first-pass metabolism of these nutraceuticals.

Keywords Gastrointestinal tract · Microbiota · Green-lipped mussel · *Perna canaliculus* · Glucosamine · Osteoarthritis · *Clostridia* sp.

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Introduction

Natural medicines are an attractive option for patients suffering from osteoarthritis (OA), (Ehrlich 2003) the most

common, debilitating musculoskeletal disorder known (Woolf and Pfleger 2003; Reginster 2002). The high rate of self-medication with natural products is due to (1) lack of an available cure (Ernst 1998) and (2) the serious adverse events associated with chronic use of prescribed drugs, particularly non-steroidal anti-inflammatory drugs (NSAIDs) (Bjordal et al. 2004) and high dose paracetamol (acetaminophen) (Garcia Rodriguez and Hernandez-Diaz 2001). However, the clinical efficacy of nutraceuticals for treating OA symptoms, such as joint pain, stiffness and limited range of motion remains collectively inconsistent, limiting recommendations for their routine use. This includes (S-adenosylmethionine (Rutjes et al. 2008), green-lipped mussel (GLM) extract (Ulbricht et al. 2009) and D-glucosamine (Wandel et al. 2010).

The GIT and microbiota combined comprise one of the most metabolically and immunologically active organs (Egert et al. 2006). Nutraceutical supplements such as GLM and GS orally administered are metabolised by gastrointestinal bacteria, potentially modifying and influencing exposure to these compounds (Laparra and Sanz 2010; Egert et al. 2006). The metabolism of GLM extract by GIT microbial species has not previously been investigated. Early in vitro studies have confirmed that commensal GIT bacteria can ferment and metabolise glucosamine (Foley et al. 2008; Koser et al. 1961; Wolfe et al. 1956). Furthermore, microbial diversity and colonisation are restricted in the proximal GIT ($\sim 10^2$ to 10^4 cfu ml^{-1}) and increases in density and species diversity within the distal GIT (10^6 to 10^8 cfu ml^{-1}) (Holzapfel et al. 1998). However, the large bowel is the most highly populated and most metabolically active region of the GIT (10^{10} to 10^{12} cfu g^{-1}) with a truly complex and diverse bacterial load estimated at more than 1,000 species with 30–40 normally predominating (Kelly et al. 2005; Guarner and Malagelada 2003).

Anaerobes outnumber aerobic bacteria by a factor of 10^2 to 10^4 within the large bowel. The genera *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus*, and *Ruminococcus* are predominant genera whereas aerobes (facultative anaerobes) such as *Escherichia, Lactobacillus, Enterobacter, Enterococcus, Klebsiella,* and *Proteus* are among the subdominant genera (Guarner and Malagelada 2003). Bacterial counts of individual species range over several orders of magnitude and the metabolic products of bacterial groups vary considerably (Egert et al. 2006). The intestinal microbiota can have marked effects on mucosal defence mechanisms (i.e. competition for mucosal colonisation and metabolic substrates plus synthesis of regulatory factors such as short-chain fatty acids and bacteriocins) and the innate and adaptive immune responses of the host, and are therefore integral to maintaining immune homeostasis within the developing and adult gut. Commensal bacteria, however, differ in their ability both to promote development of the gut-associated lymphoid tissues and to maintain its function (Kelly et al. 2005; Umesaki et al. 1999).

OA and rheumatoid arthritis (RA) can predispose patients to GIT symptoms that can be attributed to orally administered analgesics (i.e. paracetamol/acetaminophen and NSAIDs) (Chong and Wang 2008; Wolfe et al. 2000). GIT symptoms can include dyspepsia, epigastric pain and burning, bloating, early satiety, nausea and belching, mucosal ulceration and altered bowel habits (constipation/diarrhoea). In addition to analgesic and anti-inflammatory medicines inducing such GIT symptoms, altered GIT microbial growth trends may also contribute (Tana et al. 2010). We have recently reported that administration of GLM extract (3,000 mg/day) to OA patients significantly improved GIT symptoms, concurrent with significant improvement in knee pain, stiffness and function (Coulson et al. 2012). Profiling the GIT microbiota has not been previously studied in OA patients. Altered GIT microbiota profiles may be a significant factor that largely determines the therapeutic efficacy of nutraceutical supplements such as GLM and GS, in OA patients.

The GIT microbiota of OA patients may therefore be a target of nutritional intervention that influences bacterial viability, growth, and metabolic activity. Therefore, the aim of this clinical study was to assess faecal microbiota profiles in patients diagnosed with OA of the knee and then to report whether supplementation with GLM extract and GS affected the growth patterns of the faecal microbiota. Furthermore, we investigated whether bacterial growth patterns could be correlated to the therapeutic efficacy outcomes with GLM and GS.

Patients and methods

Study design

The trial was conducted according to a non-blinded, randomised, comparator-controlled, parallel-group design in one outpatient rheumatology clinic in Brisbane, QLD. The study protocol complied with the Helsinki Declaration and was approved by the Human Research Ethics Committees of the University of Queensland and The Princess Alexandra Hospital. Participants who met the inclusion criteria were then randomly assigned to one of the two treatment arms, i.e. GLM or GS. Each patient received written and verbal explanations regarding their involvement in the study before signing informed consent. They were examined before receiving their first treatment at baseline (T_0) and then re-examined at T_6 and T_{12} weeks of treatment at which time their participation was concluded.

Participant selection

The study group comprised of 40 participants diagnosed with OA in one or both knees (11 male, 29 female) who satisfied the inclusion and exclusion criteria. Patients were recruited if their knee OA was reviewed and confirmed by a Rheumatologist (PV) and they were not taking antibiotics or any form of herbal/multivitamin/nutritional supplements 4 weeks before entering this study. Participants were excluded if they had uncontrolled systemic disease, were pregnant or breast feeding or had allergies/intolerances to shellfish. They were also instructed to avoid antibiotics while enrolled in the study. All participants satisfied the decision tree format of the American College of Rheumatology (ACR) classification criteria for idiopathic clinical OA of the knee (Altman et al. 1986). The ACR clinical criteria for knee OA were fulfilled by the uniform presence of knee pain and at least three of the following six criteria: $\geq$50 years of age, morning stiffness lasting $\leq$30 min, crepitus on movement, bony tenderness, bony enlargement and no palpable warmth of synovium, or at least one of the following three criteria plus radiographic evidence of osteophytes: $\geq$50 years of age, morning stiffness lasting $\leq$30 min and crepitus on movement.

The subject's demographic data and medical history were obtained at baseline. Anthropometric measurements, blood pressure and outcome measures were performed at T_0, T_6 and T_{12} weeks. Height was measured with the subject standing barefoot using a body meter measuring tape with wall stop. Body mass was measured using calibrated scales and body mass index (BMI) was calculated using the formula: mass divided by height squared (kg/m^2); waist hip ratios were calculated by dividing the waist circumference (cm) by the hip circumference (cm). Participants were recruited from the local Brisbane area from June to November 2011 and were seen for each visit at the Princess Alexandra Hospital, Brisbane QLD. Patients were randomised for treatment with either the GLM (5 male, 16 female) or GS (6 male, 13 female) groups.

Intervention

The GLM extract sourced for this trial was GlycOmega™ PLUS from Aroma NZ Ltd, Christchurch NZ, a propriety blend of freeze-dried mussel meat (no shell), stabilised with Rosemary oil extract. The glucosamine sulphate-potassium chloride was sourced from BJP Laboratories (Brisbane, QLD). Patients were randomly allocated (non-blinded) to 3,000 mg/day (3 × 500 mg capsules b.i.d) of GlycOmega™ PLUS or 3,000 mg/day (3 × 500 mg capsules b.i.d) of GS. Capsules were dispensed in opaque white bottles for the 12 weeks duration. Compliance was checked by examining the patients' records of their daily intake of GLM or GS in provided diaries. Participants in this trial were allowed to continue taking analgesic medications (paracetamol and/or NSAIDs) as required. Participants were asked to complete two diaries that recorded daily administration of (1) GLM or GS; (2) analgesic medication and (3) knee pain experienced on a 5-point Likert scale (T_0–T_6 and T_6–T_{12}).

Randomisation

A randomisation list was generated by computer program and maintained by the investigators. Participants were sequentially assigned to a randomisation schedule number (1 = GLM or 2 = GS) at their first visit to the trial clinic post-adherence to all inclusion and exclusion criteria.

Objectives and outcomes

The primary outcome measure was to assess at T_0 and T_{12} weeks, the common GIT microbial genera.

Faecal sample collection

A morning bowel motion was collected in a faecal container by patients in their own home in a modified faecal container with the lid of the container perforated to assist sample anaerobiasis. The sample was immediately transferred into a sealed anaerobic pouch system (Oxoid, Thermo Fisher Scientific, Australia). Anaerobiasis was achieved by activating the Anaero Gen Compact (Oxoid, Thermo Fisher Scientific, Australia) before sealing the pouch. Samples were transported cold (<12 °C) to the laboratory and analysed within 48 h after collection. Samples were rejected for analysis if they were delayed during transit, had inadequate refrigeration or if the anaerobic storage during transit did not meet the criteria and remained aerobic. Patients with rejected samples were requested to resubmit another sample for analysis.

Quantitation of faecal microorganisms

All faecal samples, once removed from the anaerobic pouch system were processed within 10–15 min. A determined quantity of faecal sample (range 0.5–1.0 g) was transferred to 10 mL of 1 % glucose-saline buffer (Willis 2007). Dilution factor was determined by the difference in the weight of the glucose-saline buffer with and without the sample. One hundred and one thousand fold dilutions (beginning from 10^{-1} to 10^{-7}) of homogenised faecal samples were prepared (Thrupp 1980). A 10 and/or 1 µL amount of the appropriate dilutions were transferred each onto previously dried Columbia horse blood agar (Oxoid), chromogenic medium (Oxoid), colistin and nalidixic acid

blood selective agar (Oxoid), and chloramphenicol-gentamicin selective Sabouraud agar for aerobic incubation. Pre-reduced Columbia horse blood haemin agar and Raka Ray medium were used for anaerobic incubation. All aerobic media were incubated at 35 °C for 48 h and anaerobic media in anaerobic jars (Oxoid) for 4 days. All aerobic and anaerobic culture plates were examined under a stereomicroscope for a minimum of 20 min/plate before identification of the bacteria. Every colony from each medium was examined microscopically and quantitatively recorded. Microscopic colonies of similar morphotypes were sub-cultured onto horse blood agar to check for purity prior to identification. To determine the validity of the anaerobic transport and culture methods, two faecal samples from one of the investigators (HB), collected at different days, were processed each within 2 h after collection, and again on two different occasions, at 24 and 48 h. On each occasion the specimen was re-sealed and stored refrigerated anaerobically immediately after being processed. Results from this internal quality assurance investigation showed that there were no significant quantitative changes in either the aerobes or anaerobes processed at the three different periods. The incidence of the predominant aerobes and anaerobes remained unchanged.

Identification of faecal bacteria: MALDI-TOF MS analysis

Index bacterial colonies, from overnight purity checks, were transferred to a target polished steel plate (MSP 96 target polished steel, Bruker Daltonics Inc.) for drying under exhaust ventilation in a Class II Biohazard Hood (Gelman Sciences Australia) at room temperature. Air-dried samples were subjected to protein extraction with 1 μL 70 % formic acid (Sigma). Samples were again allowed to air dry, under exhaust ventilation, before being overlaid with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of 47.5 % ultra-pure water, 2.5 % trifluoroacetic acid, and 50 % acetonitrile). Once dried, samples were subjected to analysis using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2,000–20,000 Da at maximum laser frequency. Raw spectra were analysed, using the MALDI Biotyper 3.0 software package (Bruker Daltonik GmbH, Bremen, Germany) with default settings. Measurements were performed automatically without any user intervention.

Secondary outcome measures

Secondary outcome measures were recorded at T_0, T_6 and T_{12} weeks and included the Western Ontario McMaster Universities Arthritis Index (WOMAC) (Bellamy 2002) the Lequesne algofunctional index (Lequesne et al. 1987) the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire (Svedlund et al. 1988) and the SF-12v2™ health survey (Tucker et al. 2010). The WOMAC is a validated questionnaire containing 24 questions, designed to assess lower extremity pain and function in OA of the knee or hip by assessing severity of pain, stiffness and limitation of physical function with a maximum score of 96 (Bellamy et al. 1988). The Lequesne algofunctional index includes 10 questions for the measurement of pain, walking distance and activities of daily living (Lequesne et al. 1987). Scores for each question are added together to provide a combined disease severity score with a maximum score of 24. Scores of 1–4 are classified as mild OA, 5–7 moderate, 8–10 severe, 11–13 very severe, and greater than 14 as extremely severe OA. GIT function was assessed using the validated GSRS questionnaire which contains 15 items to evaluate abdominal pain, gastro-oesophageal reflux, indigestion, diarrhoea and constipation scored on a 7-point Likert scale. The GSRS has a maximum score of 105. General quality of life was assessed using the SF-12 questionnaire that is expressed in terms of two meta-scores: the physical component summary (PCS) and the mental component summary (MCS) (Tucker et al. 2010; Ware et al. 1996).

Assessment of safety

Any adverse events were documented by patients in their diaries kept over the 12 weeks duration of the trial. These events were recorded by giving the start and finish dates of symptoms and their severity (from 1, mild; 2, moderate and 3, severe). Medications required to treat the adverse events were also recorded in diaries provided, listing the medication's name, dose taken per day and the start and finish dates of taking the medications. Routine laboratory measurements including full blood count, serum electrolytes, liver function tests, blood glucose (non-fasting) and inflammatory markers, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were performed at time of enrolment and after 12 weeks of treatment.

Statistical analysis

All microbiology data were log10 transformed prior to statistical analysis. Normality tests were performed on bacterial data. Wilcoxon test for paired data was used to analyse the changes in bacteria within treatment groups. Kolmogorov–Smirnov tests were performed for all the scored variables to confirm the assumption of normality, justifying the use of *t* tests. Paired *t* tests were then used to compare the changes in the scores over the study period (T_0–T_{12} weeks) within the GLM and GS groups.

Independent group *t* tests were used to test for differences in changes in the scores between the GLM and GS groups. The scoring analysis was by assessment of the difference between groups and within groups in the change from baseline to 12 weeks, in the intention to treat (ITT) population (i.e. including all randomised patients with at least 1 efficacy assessment after randomisation). The significance level was set at $p < 0.05$. Analgesic usage was assessed in patients who completed, or partially completed, the 12 weeks treatment course. The mean number of medications per 7-day period was tabulated for descriptive purposes. A similar graph was produced for the amount of pain reported, also as a 7-day average over 12 weeks of the study.

Results

The demographic and clinical characteristics of the randomised patients were comparable at baseline (Table 1). The majority of patients were female, being 74 % of the cohort. The mean age was 58.6 ± 8.9 years and 89 % of patients were overweight (BMI >25 kg/m^2) and 58 % were obese (BMI >30 kg/m^2). The mean duration of knee OA was 9.4 ± 8.3 years. For the overall cohort mean WOMAC score at baseline was 36.3 and the Lequesne index score 11.7 indicating chronic disease with moderate symptom severity (Table 1). Four patients withdrew from the trial with 2 patients providing their first diary and completing the questionnaires at T_6 weeks yielding a total of 38 participants (10 male, 28 female). Twenty-one patients in the GLM group and 17 in the GS group provided data for an analysis of Lequesne, WOMAC, GSRS and SF-12 scores (Fig. 1). Patient numbers for bacterial analysis differed slightly, the GLM group providing 21 faecal samples for analysis at baseline but only 18 samples at week 12 (due to samples being lost in transit to the analytical laboratory). The GS group provided 17 faecal samples at both baseline and week 12.

Table 1 Demographic and baseline clinical characteristics of patients in the intention-to-treat population

Characteristics	Intervention	
	Green-lipped mussel group ($n = 21$)	Glucosamine sulphate group ($n = 17$)
Women, no (%)	16 (76)	12 (71)
Age (years)	56.7 ± 8.9	60 ± 8.6
BMI (kg/m^2)	31.3 ± 6.1	30.2 ± 4.8
Weight (kg)	90.1 ± 17.1	87.1 ± 15.4
Waist:hip ratio	0.9 ± 0.1	0.9 ± 0.1
Duration of knee OA (years)	7.5 ± 5.9	11.4 ± 9.5
Lequesne index	12.2 ± 3.4	11.1 ± 2.6
WOMAC		
Total	37.4 ± 13.8	34.9 ± 10.8
Pain	8.0 ± 2.8	7.5 ± 2.7
Stiffness	4.2 ± 1.5	3.6 ± 1.3
Function	25.2 ± 10.2	23.8 ± 7.8
GSRS	34.4 ± 19.5	24.3 ± 9.9

Except where indicated otherwise, values are represented as mean ± SD

Weight, BMI and waist-to-hip ratios did not change significantly within each group between time points. Current and stable medications were continued throughout the trial. The type of medications and number of patients taking them included cholesterol lowering agents ($n = 10$), anti-diabetic agents ($n = 4$), diuretic ($n = 1$), anti-hypertensive medications ($n = 8$), anti-inflammatory medications ($n = 3$), proton pump inhibitors ($n = 11$), anti-depressant medications ($n = 7$), thyroid medications ($n = 3$), anti-histamine medication ($n = 5$), anti-coagulant medications ($n = 9$), benzodiazepines ($n = 2$), medications for migraine ($n = 2$), gout medication ($n = 1$). We also importantly note that these medications prescribed to participants by their clinicians for management of chronic diseases had been administered at length prior to participating in the trial and remained as such during the trial. Therefore, we can safely deduce that baseline microbiota readings should not have changed over the 12 weeks due to these prescribed medications. This further substantiating that any changes that occurred at 12 weeks were not influenced by these pharmaceuticals.

Prescribed antibiotics, but not within 4 weeks preceding trial commencement, was reported by 9 patients while 26 patients reported recent influenza vaccinations. The rate of compliance with GLM or GS intake as per the protocol (3 caps b.i.d for 12 weeks) was 34 % of the participants not missing any doses, while the remaining 66 % missed 1 or more doses over the 12-week study period.

Four patients from the original 40 did not complete the study namely, 1 male from the GLM group and 2 males and 1 female from the GS group. Drop-outs were due to either lack of interest or an adverse event unrelated to the GLM or GS. Two of the male dropouts (1 GLM, 1 GS) did return their first diaries and completed week 6 questionnaires. The data was included for analysis as intention-to-treat with one also providing a faecal sample for bacterial analysis at week 12.

Faecal analysis

Of the 73 faecal samples analysed, 12 genera and 137 species were recovered (data not shown). The mean viable count [colony forming units (cfu)/g] of the total aerobic and anaerobic microbial flora at baseline was not significantly different between the two treatment groups. The

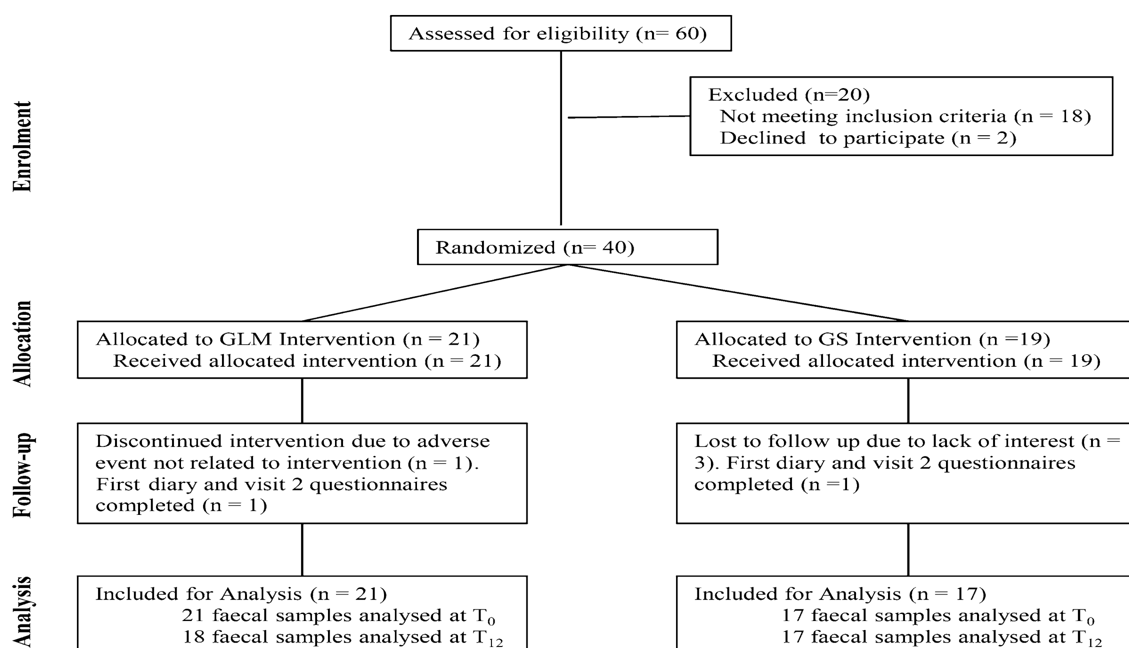

Fig. 1 CONSORT diagram of participant inductions

mean viable counts and p values (determined by the Wilcoxon test) of aerobic and anaerobic species are presented in Table 2. A number of GIT bacterial species in these OA participants at baseline demonstrated variation from the GIT species in the control data ($n = 177$) with no gastrointestinal symptoms (data on file, Bioscreen). The viable count of microorganisms that were increased from the control data (at baseline) included *Enterococcus, Streptococcus, Staphylococcus, Eubacterium, Lactobacillus, Bifidobacterium* and *Clostridium*. The bacterial counts of these microorganisms remained high, at week 12 when compared to the control data. However, *Clostridial* and *Staphylococcus* species were observed to decrease from baseline to 12 weeks in both treatment groups.

Within-group faecal microbial analysis did not demonstrate statistically significant changes within any of the bacterial species from T_0 to T_{12} weeks after supplementation with GLM or GS. Further analyses of the data did, however, demonstrate an effect on the growth trends (i.e. decreased or increased) among the microbiota profiles that differed between the two treatment groups. Overall, the GLM-treated group demonstrated a trend towards decreasing *Enterococcus* sp., *Staphylococcus* sp. *Clostridium* sp. and yeasts and an increased *Lactobacillus* sp., *Bifidobacterium* sp., *Eubacterium* sp., *Bacteroides* sp. and *Streptococcus* sp. By comparison the GS-treated group demonstrated a trend towards decreasing *Staphylococcus* sp., *Bacteroides* sp. and *Clostridium* sp. and increased *Coliforms, Streptococcus* sp., *Eubacterium* sp. and *Lactobacillus* sp. The most prevalent species detected within each genera were as follows: *Coliforms* (*Escherichia coli, Klebsiella pneumoniae*); *Enterococcus* (*E. faecalis, E. faecium*); *Streptococcus* (*S. mutans, S. parasanguinis, S. salivarius*); *Staphylococcus* (*S. aureus, S. epidermidis, S. capitis*); Yeasts (*Candida albicans*); *Bacteroides* (*B. vulgates, B. ovatus, B. fragilis, B. uniformis, B. thetaiotaomicron, B. stercoris*); *Eubacterium* (*Collinsella aerofaciens*); *Lactobacillus* (*L. acidophilus, L. gasseri, L. paracasei, L. fermentum*); *Bifidobacterium* (*B. longum, B. animalis, B. adolescentis, B. bifidum*); *Clostridium* (*C. innocuum, C. tertium*) with *C. perfringens* detected in one patient at baseline.

Paired sample t tests were performed on the Lequesne, WOMAC (total and subscores), GSRS, and two component SF-12 scores (PCS and MCS) between T_0 and T_6 weeks and T_0 and T_{12} weeks for each treatment group. The results are reported in Table 3. Both the GLM and GS-treatment groups demonstrated statistical significance for the Lequesne and WOMAC total and subscores between all intervals, $p < 0.001$ and $p = 0.001$, respectively. The GLM group also demonstrated statistical significance for GSRS scores ($p = 0.02$) and borderline significance for the GS group ($p = 0.044$). The incidence of GSRS symptoms is reported in Table 4. The PCS scores for both the GLM ($p = 0.004$) and GS ($p = 0.001$) groups improved significantly from baseline to week 12, whereas the MCS scores showed no statistical improvement for both the GLM ($p = 0.20$) or GS ($p = 0.70$) groups.

Analgesic medication was allowed and recorded daily by the participants over the 12-week trial period. The need for analgesic medications decreased in the GLM group with a mean consumption at baseline of 1.07 ± 1.62

Table 2 Mean viable counts (cfu/g) and Wilcoxon signed ranks test (p value) of faecal aerobes and anaerobes in the green-lipped mussel and glucosamine sulphate groups at baseline and week 12

Organism	Normal range[a]	Green-lipped mussel group			Glucosamine sulphate group		
		Baseline ($n = 21$)	Week 12 ($n = 18$)	p value	Baseline ($n = 17$)	Week 12 ($n = 17$)	p value
Total bacterial count	1×10^9 to 1×10^{12}	2.31×10^{10}	3.29×10^{10}	0.36	4.04×10^{10}	3.10×10^{10}	0.72
Total aerobes	1×10^7 to 1×10^8	7.17×10^7	8.83×10^7	0.71	6.98×10^7	1.02×10^8	0.35
Coliforms	7×10^6 to 9×10^7	4.38×10^7	5.82×10^7	0.68	6.01×10^7	7.52×10^7	0.77
Enterococcus	$<5 \times 10^5$	9.32×10^6	5.75×10^6	0.48	4.86×10^6	8.41×10^6	0.64
Streptococcus	$<3 \times 10^5$	1.12×10^7	2.84×10^7	0.35	1.76×10^7	2.98×10^7	0.83
Staphylococcus	$<2 \times 10^5$	4.51×10^6	1.97×10^4	1.00	7.03×10^5	4.78×10^4	0.36
Yeast	$<1 \times 10^4$	9.49×10^3	3.46×10^3	0.13	3.85×10^3	3.92×10^5	0.75
Total anaerobes	1×10^8 to 1×10^{12}	2.30×10^{10}	3.28×10^{10}	0.35	4.03×10^{10}	3.09×10^{10}	0.74
Bacteroides	9×10^7 to 9.5×10^{11}	1.31×10^{10}	1.82×10^{10}	0.23	3.05×10^{10}	1.98×10^{10}	0.27
Prevotella	$<5 \times 10^8$	ND	ND		1.90×10^8	ND	
Eubacterium	$<1 \times 10^9$	6.36×10^9	1.24×10^{10}	0.39	5.02×10^9	7.60×10^9	0.25
Lactobacillus	5×10^5 to 1×10^7	8.06×10^8	8.44×10^8	0.75	1.87×10^8	8.27×10^8	0.76
Bifidobacterium	5×10^5 to 5×10^8	3.83×10^9	4.19×10^9	0.51	7.75×10^9	5.58×10^9	0.24
Clostridium	$<5 \times 10^8$	1.91×10^9	8.11×10^8	0.70	1.84×10^9	9.95×10^8	0.94

ND not detected

[a] Normal range determined by normal healthy population (Bioscreen data)

Table 3 Mean (95 % confidence interval) change from baseline in secondary outcome measures. Mean (95% confidence interval) change from baseline in secondary outcome measures and differences between groups

Outcomes	Green-lipped mussel ($n = 21$)	Glucosamine sulphate ($n = 17$)	Difference between groups
GSRS			
Change T_0–T_6	−10.2 (2.6, 17.9)	−3.8 (−0.7, 8.3)	
Change T_0–T_{12}	−10.4 (1.8, 18.9)	−3.9 (0.1, 7.8)	−6.4 (−15.6, 2.7)
p*	0.02	0.044	0.162
Lequesne index			
Change T_0–T_6	−3.7 (2.1, 5.3)	−2.9 (1.3, 4.6)	
Change T_0–T_{12}	−4.2 (2.2, 6.1)	−3.3 (1.6, 4.9)	−0.9 (−3.4, 1.7)
p	<0.001	0.001	0.5
WOMAC (total)			
Change T_0–T_6	−16.4 (10.6, 22.3)	−10.4 (3.1, 17.6)	
Change T_0–T_{12}	−18.9 (12.7, 25.1)	−14.8 (7.3, 22.2)	−4.1 (−13.4, 5.1)
p	<0.001	0.001	0.372
WOMAC (pain)			
Change T_0–T_6	−4.0 (2.5, 5.4)	−2.4 (0.2, 4.5)	
Change T_0–T_{12}	−4.9 (3.4, 6.3)	−3.3 (1.5, 5.1)	−1.6 (−3.7, 0.6)
p	<0.001	0.001	0.157
WOMAC (stiffness)			
Change T_0–T_6	−1.5 (0.8, 2.3)	−1.1 (0.5, 1.7)	
Change T_0–T_{12}	−2.4 (1.6, 3.1)	−1.2 (0.7, 1.8)	−1.1 (−2.0, 0.2)
p	<0.001	<0.001	0.02
WOMAC (physical)			
Change T_0–T_6	−11.0 (6.6, 15.3)	−6.9 (1.8, 12.0)	
Change T_0–T_{12}	−11.8 (7.2, 16.3)	−10.2 (4.7, 15.6)	−1.6 (−8.3, 5.2)
p	<0.001	0.001	0.639

p values and 95 % confidence intervals of the differences between groups were derived by independent samples t tests

GSRS gastrointestinal symptom rating score

* p values represent change in score from T_0–T_{12}

Table 4 Gastrointestinal complaints: number of patients in both treatment groups at baseline and week 12 and range of severity according to the GSRS 7-point Likert scale

Gastrointestinal symptom[a]	Green-lipped mussel group		Glucosamine sulphate group	
	Baseline	Week 12	Baseline	Week 12
Reflux	5 (moderate–severe)	2 (mild–moderate)	4 (mild–severe)	1 (severe)
Constipation	6 (mild–severe)	3 (mild–moderate)	2 (mild–moderate)	1 (mild)
Diarrhoea	5 (mild–very severe)	3 (mild–very severe)	2 (mild–moderate)	0
Heart burn	5 (moderate–severe)	2 (mild–moderate)	5 (mild–severe)	2 (moderate–severe)
Nausea	6 (mild–mod/sev)	1 (moderate)	2 (mild–mod/sev)	1 (mod/sev)
Bloating	8 (mild–very severe)	5 (mild–moderate)	7 (mild–severe)	3 (mild)
Abdominal pain	6 (mild–severe)	4 (mild–severe)	4 (mild–severe)	2 (mild–moderate)
Flatulence	9 (mild–very severe)	6 (mild–severe)	5 (mild–severe)	5 (mild–severe)
Burping	7 (mild–very severe)	4 (mild–moderate)	3 (mild–severe)	1 (mild)
Rumbling	6 (mild–mod/sev)	5 (mild–mod/sev)	5 (mild–mod/sev)	4 (mild–moderate)

mod/sev: moderate/severe symptoms, rumbling refers to stomach rumbling

[a] Complaints reported in the Gastrointestinal Symptom Rating Scale (GSRS)

tablets/capsules per day to 0.66 ± 1.10 tablets/capsules at week 12. The intake of analgesic medication in the GS group, however, did not change with a mean consumption at baseline of 0.48 ± 0.80 tablets/capsules per day to 0.47 ± 1.02 tablets/capsules per day at week 12 (Fig. 2). Only 18 % of patients in the GS group did not use analgesic medication at all, compared with 29 % in the GLM group. Analgesic medication and doses per capsule included panadol (paracetamol 500 mg), panadol osteo (paracetamol 665 mg), panadeine forte (paracetamol 500 mg + codeine phosphate 30 mg), and various NSAIDs (aspirin 100 mg; indomethacin 25 mg; diclofenac sodium 50 mg; ibuprofen 200 mg).

Adverse events

The number of adverse events reported during the trial period was similar in both groups and is presented in Table 5. The most frequent adverse events were upper respiratory tract infections (URTI) for which seven patients (6 GLM, 1 GS) took antibiotics over a period of 4–26 days while enrolled within the study. Other events included gastrointestinal disturbances for which 1 patient in the GS group took probiotics for 21 days (pharmacist recommendation), headaches, migraines, injuries, angina and hip surgery. None of the adverse events were deemed related to the GLM or GS preparations.

A subsequent sub-analysis of faecal microbiota profiles at 12 weeks after correcting for the antibiotic ($n = 7$) and probiotic ($n = 1$) use, showed that the remaining participants' microbiota profile trends did not deviate from the uncorrected trend in both groups (compare Tables 2 and 6). Specifically participants who were prescribed an antibiotic (6 in GLM and 1 GS group) demonstrated at 12 weeks a trend towards an increase in *Enterococcus, Streptococcus, Eubacterium* and *Lactobacillus* species. Decreased growth was noted for *Coliforms*, Yeasts, *Bifidobacterium* and *Clostridia* species. For the sole participant who was prescribed a single-strain probiotic (containing 3 *Lactobacillus* sp.)

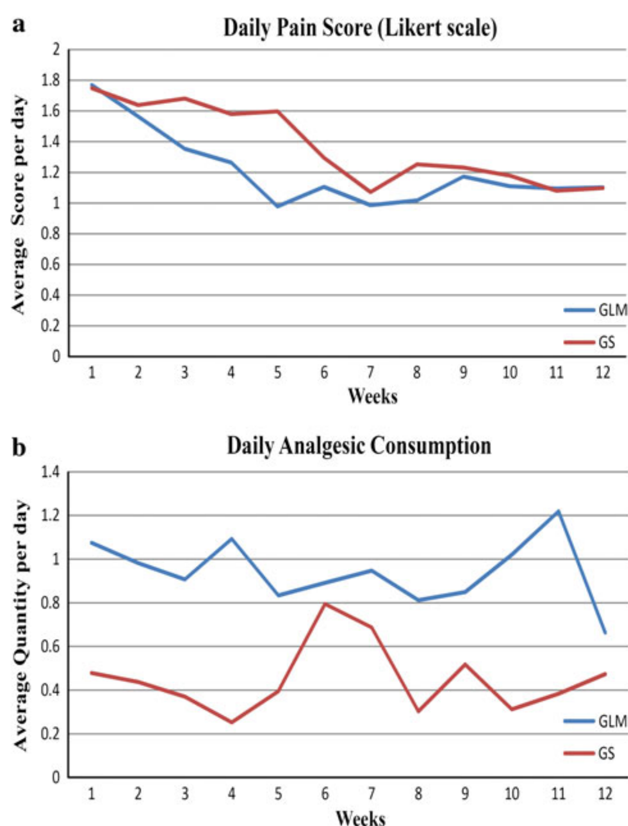

Fig. 2 **a** Average daily pain scores and **b** average analgesic medication administered for GLM and GS groups

Table 5 Summary of adverse events experienced by patients at any time during the treatment period

Adverse event	No. of patients experiencing an adverse event	
	Green-lipped mussel group	Glucosamine sulphate group
Gastrointestinal symptoms		
Reflux	1	1
Constipation	2	2
Diarrhoea	0	1
Heart burn	1	1
Nausea	3	0
Bloating	1	0
Abdominal pain	0	1
Infections		
Respiratory tract infections	8	4
Gastroenteritis	2	1
Cutaneous infections	1	0
Respiratory Symptoms		
Coughing and associated symptoms	3	2
Allergies	1	2
Neurological symptoms		
Headache	4	5
Migraine	1	2
Insomnia	1	0
Musculoskeletal symptoms		
Back/neck pain	0	4
Shoulder pain	3	1
Injuries		
Fall	1	0
Other	2	2
Cardiovascular symptoms		
Angina	1	0

showed at 12 weeks an increased trend in *Coliforms, Eubacterium, Lactobacillus* sp. and decreased growth of *Streptococcus and Clostridia* sp. (Table 6).

Safety measures

Baseline blood analyses indicated that a number of liver function enzymes were elevated. Gamma-glutamyl transferase (GGT) was increased in two patients (1 GLM, 1 GS), mean ± SD, 54.3 ± 9.8 U/L (reference range <38 U/L) and alanine transaminase (ALT) was increased in three patients (2 GLM, 1 GS), mean ± SD, 54.3 ± 13.7 U/L (reference range <34 U/L) and aspartate transaminase (AST) was increased in three patients (3 GLM), mean ± SD, 41.7 ± 3.1 U/L (reference range <31 U/L). These patients, however, were taking a number of medications for hyperlipidaemia, hypertension, diabetes, gastric reflux and depression/anxiety that are known to elevate liver enzymes (Gillett and Norrell 2011; Chitturi and George 2002). At 12 weeks, GGT was elevated in four participants (2 GLM, 2 GS); mean ± SD, 49.8 ± 5.9 and ALT was elevated in two participants (2GLM), mean ± SD, 40.5 ± 0.7 U/L. AST levels returned to normal range in the three patients by 12 weeks. Non-fasting blood glucose levels were elevated in three patients (2 GLM, 1 GS) at the end of treatment (mean ± SD, 9.3 ± 1.9 mmol/L), however; all three patients had type II diabetes mellitus prior to enrolment and were receiving medication for their diabetes. Mean CRP values differed slightly between the groups at baseline and 12 weeks with the GS group demonstrating a decrease in CRP (mean ± SD, $T_0 = 4.69 \pm 4.76$ mg/L; $T_{12} = 2.78 \pm 1.28$ mg/L) compared to GLM (mean ± SD, $T_0 = 4.62 \pm 4.69$ mg/L; $T_{12} = 5.22 \pm 4.75$ mg/L). The GS group also demonstrated a decrease in ESR (mean ± SD, $T_0 = 19.06 \pm 14.53$ mm/h; $T_{12} = 16.92 \pm 12.51$ mm/h) compared to the GLM group (mean ± SD, $T_0 = 14.65 \pm 7.15$ mm/h; $T_{12} = 16.89 \pm 8.95$ mm/h). There were no significant changes in the other routine laboratory parameters. Blood pressure remained stable throughout the treatment period in both the GLM (mean ± SD, T_0 = systolic 131.0 ± 13.51 mmHg and diastolic 79.06 ± 9.97 mmHg; T_{12} = systolic 132.69 ± 16.1 mmHg and diastolic 79.46 ± 11.77 mmHg) and GS groups (mean ± SD, T_0 = systolic 129.0 ± 11.69 mmHg and diastolic 75.38 ± 11.75 mmHg; T_{12} = systolic 128.74 ± 10.57 mmHg and diastolic 76.0 ± 8.05 mmHg).

Discussion

This study evaluated some of the commonly encountered GIT microbial genera in patients with OA before and after treatments with GLM or GS. Specifically, we have assessed how GLM and GS, over a 12-week period, might alter common bacterial growth patterns in the GIT. This clinical study has provided an alternate hypothesis that could explain the variable and contentious therapeutic effects GLM and GS have elicited in clinical trials (Brien et al. 2008; Towheed et al. 2005). To this end, GIT dysbiosis may have a significant role to play in nutraceutical efficacy for OA. We observed a significant improvement in GSRS scores, primarily, in the GLM-treated group, corroborating previous observations (Coulson et al. 2012). This study also confirmed that both GLM and GS each significantly attenuated knee joint pain, stiffness and improved joint flexibility and function.

Several methodological issues and industry bias have been suggested as contributing factors that have led to inconsistent clinical findings when evaluating GLM and GS efficacy (Brien et al. 2008; Towheed et al. 2005). The administration of analgesic medications to rescue pain, or the exclusion of their use, may be an important factor that

Table 6 Subanalysis of mean viable counts (cfu/g) of faecal aerobes and anaerobes in the green-lipped mussel, glucosamine sulphate, antibiotic and probiotic groups at baseline and week 12

Organism	Normal range[a]	Green-lipped mussel group		Glucosamine sulphate group		Administered			
						Antibiotics		Probiotics	
		Baseline ($n = 15$)	Week 12 ($n = 12$)	Baseline ($n = 15$)	Week 12 ($n = 15$)	Baseline ($n = 7$)	Week 12 ($n = 7$)	Baseline ($n = 1$)	Week 12 ($n = 1$)
Total bacterial count	1×10^9 to 1×10^{12}	2.54×10^{10}	3.93×10^{10}	4.34×10^{10}	3.27×10^{10}	1.77×10^{10}	1.98×10^{10}	1.52×10^{10}	1.75×10^{10}
Total aerobes	1×10^7 to 1×10^8	5.60×10^7	1.07×10^8	7.59×10^7	8.57×10^7	9.96×10^7	9.06×10^7	2.27×10^7	1.17×10^8
Coliforms	7×10^6 to 9×10^7	1.93×10^7	6.70×10^7	6.89×10^7	8.03×10^7	9.02×10^7	3.47×10^7	1.32×10^7	7.80×10^7
Enterococcus	$<5 \times 10^5$	1.80×10^7	6.58×10^6	6.18×10^6	6.27×10^5	2.25×10^6	3.96×10^6	4.04×10^5	3.91×10^7
Streptococcus	$<3 \times 10^5$	1.09×10^7	3.65×10^7	1.73×10^7	1.04×10^7	1.84×10^7	6.25×10^7	9.08×10^6	ND
Staphylococcus	$<2 \times 10^5$	4.51×10^6	1.97×10^4	7.03×10^5	4.78×10^4	ND	ND	ND	ND
Yeast	$<1 \times 10^4$	8.52×10^3	4.19×10^3	4.94×10^3	5.22×10^5	1.00×10^4	2.00×10^3	1.01×10^2	1.30×10^2
Total anaerobes	1×10^8 to 1×10^{12}	2.53×10^{10}	3.92×10^{10}	4.34×10^{10}	3.26×10^{10}	1.76×10^{10}	1.97×10^{10}	1.52×10^{10}	1.74×10^{10}
Bacteroides	9×10^7 to 9.5×10^{11}	1.26×10^{10}	2.05×10^{10}	3.33×10^{10}	2.09×10^{10}	1.46×10^{10}	1.43×10^{10}	4.04×10^9	3.90×10^9
Prevotella	$<5 \times 10^8$	ND	ND	1.62×10^9	ND	ND	ND	ND	ND
Porphyromonas	$<5 \times 10^8$	ND	ND	1.80×10^9	ND	ND	ND	ND	ND
Eubacterium	$<1 \times 10^9$	8.14×10^9	1.61×10^{10}	5.40×10^9	7.80×10^9	2.21×10^9	4.24×10^9	ND	5.20×10^9
Lactobacillus	5×10^5 to 1×10^7	1.08×10^9	4.50×10^8	2.02×10^8	2.64×10^8	9.24×10^7	1.42×10^9	1.82×10^7	6.72×10^9
Bifidobacterium	5×10^5 to 5×10^8	4.85×10^9	5.65×10^9	8.22×10^9	6.13×10^9	1.07×10^9	1.73×10^8	ND	ND
Clostridium	$<5 \times 10^8$	2.04×10^9	9.48×10^8	1.98×10^9	9.95×10^8	1.01×10^9	5.38×10^8	1.01×10^9	ND

ND not detected

[a] Normal range determined by normal healthy population (Bioscreen data)

explains the null results reported for OA. Analgesic medications administered by the cohort in this clinical study showed a decreased trend with study progress. This was observed to be greater for the GLM group than for the GS group. We hence hypothesise that bacterial metabolism of nutraceutical compounds in the GIT may generate metabolites that further confound an equivocal understanding of the mechanism of action of nutritional supplement therapies. This may be especially relevant for analgesic medications used and administered by patients diagnosed with OA, given that GIT dysbiosis may play a significant role in sustaining inflammatory sequelae both locally and systemically (Vitetta et al. 2012).

GIT microbial profiles have not yet been hitherto considered relevant in assessing symptom attenuation in OA patients. In this study we partly demonstrate that the GIT microbiome may provide new insights into the control of gastrointestinal pro-inflammatory and/or adverse immune responses in OA. Therapeutic interventions modulating a poorly regulated GIT inflammatory response might be of benefit in musculoskeletal diseases. We observed that an improvement in GSRS scores correlated with improvement in OA joint pain and flexibility in this study and this would tend to support this nexus. Hence a consequent biologically plausible postulate for GLM and GS efficacy in this study may intimately involve the rescue of GIT analgesic promoted dysbiosis by high doses of GLM and GS with concurrently less use of analgesic medications over the study period.

Studies with germ-free mice have demonstrated a link between segmented filamentous bacteria—*Clostridium*-related, Gram-positive bacteria and adherence to GIT epithelial cells and generation of TH17 cells (Smith et al. 2007; Gaboriau-Routhiau et al. 2009). The significance of these studies is that segmented filamentous bacteria (as a member of the *Clostridia* sp.) may trigger T-cell driven GIT inflammation (Stepankova et al. 2007) and arthritis (Wu et al. 2010) in mouse models. *Clostridia* sp. also induce T regulatory lymphocytes that play a critical role in the maintenance of immune homeostasis (Atarashi et al. 2011). In this clinical trial, the *Clostridium* sp. was observed to be reduced in both treatment groups over the 12-week period. Concurrently the decrease in the *Clostridium* sp. count in both groups was paralleled to reductions in knee OA symptoms. Thus, the results of this clinical study suggest that re-regulation of the GIT pro-inflammatory response may have occurred.

Investigating nutraceuticals such as GLM and GS can provide further clues as to how the GIT microbiota may normalise dysregulated inflammatory responses within the GIT that produce systemic responses elsewhere in the host. There is no rigidly defined '*normal*' reference range for mucosal bacterial species within the human GIT. Rather, the interpretation is by common growth trends with a large number of microbiota species shared between individuals. Hence, understanding the precise molecular interactions that encourage bacterial species to induce immune-stimulation by promoting either pro- or anti-inflammatory activity will be an important step towards manipulating the GIT microbiota for achieving therapeutic responses both within and beyond the gut (Barnes and Powrie 2011).

We also note that glucosamine has been reported to have poor bioavailability after oral administration (Aghazadeh-Habashi et al. 2002), and as such we postulate that the GIT, rather than the liver, would appear to be the most likely site for the first-pass effect. A large number of GIT microbial species are known to ferment glucosamine, including *Lactobacilli* (*L. casei, L. plantarum, L. acidophilus,* and *L. leichmanii*), *Streptococcus, Staphylococcus* and *Leuconostoc* (Koser et al. 1961) and *Escherichia coli, Enterococcus faecalis, Proteus vulgaris* and *Bacillus coli* (Lutwak-Mann 1941; Wolfe and Nakada 1956).

We surmise then that the GIT is often a forgotten organ when considering the pathogenesis of musculoskeletal diseases. Future clinical studies that include a placebo and GIT microbiota profiling may further demonstrate and elucidate clear differences in GIT microbiota shifts that can be significantly correlated to the rescue of GIT dysbiosis. Such studies may thus further strengthen the evidence-base of GLM and GS efficacy in OA symptom improvement and management.

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Conflict of interest GLM extract capsules were donated by Aroma New Zealand Ltd who had no involvement in the collection, analysis or interpretation of the data, writing the report or the decision to submit the paper for publication.

Ethical approval and clinical trial registration Approval for this prospective study was obtained from the Ethics Committee of The University of Queensland and Princess Alexandra Hospital Human Research Ethics Committees. The clinical trial was registered with the Australian and New Zealand Clinical Trail Registry [ANZCTRN: 12611000517976].

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