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(54) Titre : BACTERIES PROBIOTIQUES RECOMBINANTES POUR LA PREVENTION ET LE TRAITEMENT DU
 SYNDROME ABDOMINAL INFLAMMATOIRE (IBD) ET DU SYNDROME DU COLON IRRITABLE (IBS)
 (54) Title: RECOMBINANT PROBIOTIC BACTERIA FOR THE PREVENTION AND TREATMENT OF INFLAMMATORY
 BOWEL DISEASE (IBD) AND IRRITABLE BOWEL SYNDROME (IBS)

(57) **Abrégé/Abstract:**

The present invention relates to the general field of therapy of Inflammatory Bowel Disease (IBD) and/or Irritable Bowel Syndrome (IBS). Thus, the invention relates to a molecule selected from the trappin-2 protein or an active fraction of the trappin-2 protein, a member of the WAP family proteins or an active fraction of a member of the WAP family proteins or a member of the Serpin family proteins or an active fraction of a member of the Serpin family proteins for the treatment of Irritable Bowel Syndrome (IBS). The invention also relates to a recombinant food-grade bacterium comprising a gene selected from a gene coding for the trappin-2 protein or an active fraction of the trappin-2 protein, a gene coding for a member of the WAP family proteins or an active fraction of a member of the WAP family proteins, or a gene coding for a member of the serpin family proteins or an active fraction of a member of the serpin family proteins.



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(54) Title: RECOMBINANT PROBIOTIC BACTERIA FOR THE PREVENTION AND TREATMENT OF INFLAMMATORY BOWEL DISEASE (IBD) AND IRRITABLE BOWEL SYNDROME (IBS)

(57) Abstract: The present invention relates to the general field of therapy of Inflammatory Bowel Disease (IBD) and/or Irritable Bowel Syndrome (IBS). Thus, the invention relates to a molecule selected from the trappin-2 protein or an active fraction of the trappin-2 protein, a member of the WAP family proteins or an active fraction of a member of the WAP family proteins or a member of the Serpin family proteins or an active fraction of a member of the Serpin family proteins for the treatment of Irritable Bowel Syndrome (IBS). The invention also relates to a recombinant food-grade bacterium comprising a gene selected from a gene coding for the trappin-2 protein or an active fraction of the trappin-2 protein, a gene coding for a member of the WAP family proteins or an active fraction of a member of the WAP family proteins, or a gene coding for a member of the serpin family proteins or an active fraction of a member of the serpin family proteins.



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**Recombinant probiotic bacteria for the prevention and treatment of Inflammatory
Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS)**

5 **FIELD OF THE INVENTION:**

The present invention relates to the general field of therapy of gut inflammatory diseases such as Inflammatory Bowel Diseases (IBD), pulmonary diseases such as cystic fibrosis and broncho-pulmonary chronic obstructive diseases (BPCO), inflammatory articular disease (such as osteoarthritis), inflammatory urogenital disease, and diseases associated with chronic visceral pain symptoms, such as Irritable Bowel Syndrome (IBS).
10

BACKGROUND OF THE INVENTION:

15 The treatment of chronic inflammatory disorders such as IBD represents a major medical challenge as they afflict several millions of persons. Its highest incidence is among developed countries and has been increasing steadily over the past 3 decades. Current therapies for IBD strongly need to be improved, a high percentage of patients (between 20 and 40 %) being resistant to any forms of treatments, severe side effects and high costs being
20 also associated to the currently available drugs (glucocorticoids and monoclonal antibody therapies). In addition, the mechanisms involved in the pathogenesis of IBD are not fully understood, and the development of more effective treatments or even cures for IBD depends upon better understanding the regulation of the inflammatory response. Several studies have demonstrated a crucial role for proteases in the maintenance of chronic inflammatory
25 response of the gastrointestinal tract (GIT) [Vergnolle, N. 2005.; Cenac, N. et al., 2007; Hyun, E., et al., 2008; Vergnolle, N., et al., 2004]. Therefore, endogenous protease inhibitors seem to be crucial to the control of intestinal inflammatory responses.

Based on this knowledge, the inventors propose that delivery of those protease
30 inhibitors into the GIT, could be used for the treatment of an IBD and/or irritable Bowel syndrome (IBS).

The use of probiotics for the treatment of IBD has now been proposed for several years and different studies have reported some beneficial effects of these probiotic bacteria tested alone or in combination [Hedin, C. et al., 2007; Sartor, R. B. 2004]. The strategy of

using recombinant non-pathogenic food-grade bacteria as delivery vehicles of anti-inflammatory molecules at the mucosal level has already been used to deliver the anti-inflammatory cytokine IL-10 [Steidler, L., et al., 2000]. Phase I clinical trials have demonstrated that orally given *Lactococcus lactis* strain expressing IL-10 cytokine, was safe
5 as no serious side-effects occurred in those patients [Baat,H., et al., 2006]. However, the decreased disease activity in Crohn's disease patients treated with IL-10 recombinant *L. lactis* was somehow limited. This limited efficacy could be explained by the fact that IL-10 delivery has always been reported to have only discrete beneficial effects against the development of colitis [Baat, H. et al., 2003]. A better choice in the nature of the anti-inflammatory molecule
10 to be delivered by *L. lactis*, could thus considerably improve the efficacy of treatment. Here, the inventors propose to use a food-grade bacterium to express and deliver anti-protease trappin-2 into the gut.

SUMMARY OF THE INVENTION:

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The invention is based on the discovery that the use of a food-grade bacterium to deliver an anti-inflammatory molecule such as trappin-2 provides a safety and better efficiency than existing treatments. Thus, the invention relates to a molecule selected from the trappin-2 protein or an active fraction of the trappin-2 protein, a member of the WAP family
20 proteins or an active fraction of a member of the WAP family proteins or a member of the Serpin family proteins or an active fraction of a member of the Serpin family proteins for the treatment of Irritable Bowel Syndrome (IBS).

A further object of the invention relates to a recombinant food-grade bacterium comprising a gene selected from a gene coding for the trappin-2 protein or an active fraction
25 of the trappin-2 protein, a gene coding for a member of the WAP family proteins or an active fraction of a member of the WAP family proteins, or a gene coding for a member of the serpin family proteins or an active fraction of a member of the serpin family proteins.

Another aspect of the invention relates to a therapeutic composition comprising a recombinant food-grade bacterium as defined above.

30

DETAILED DESCRIPTION OF THE INVENTION:

Definitions:

As used herein, the term “trappin-2” (also known as elafin, elafin-specific inhibitor (ESI) or SKALP for skin anti-leucoprotease) of the WAP family, denotes a low molecular weight (9.9 kDa) inhibitor of HNE (human neutrophil elastase) and proteinase 3, which is secreted in the respiratory tract [Sallenave et al., 1991 and 1993]. Along with (A1-Pi) and SLPI, trappin-2 comprises an integral part of the ‘anti-elastase shield’ in the lung. An exemplary sequence for human trappin-2 gene is deposited in the database Genbank under accession number S58717.

As used herein, the term “WAP family” for “Whey Acidic Protein” denotes a family of protein containing the trappin-2, and the ps20.

As used herein, the term “Serpine family” for SERine Protease INhibitors denotes a family of serine proteinase inhibitors which are similar in amino acid sequence and mechanism of inhibition, but differ in their specificity toward proteolytic enzymes. This family includes alpha 1-antitrypsin (A1-Pi), angiotensinogen, ovalbumin, antiplasmin, alpha 1-antichymotrypsin, thyroxine-binding protein, complement 1 inactivators, antithrombin III, heparin cofactor II, plasminogen inactivators, gene Y protein, placental plasminogen activator inhibitor, and barley Z protein. This family does not include the secretory leukocyte proteinase inhibitor (SLPI) [Thierry Moreau et al., 2008]. Some members of the serpin family may be substrates rather than inhibitors of serine endopeptidases, and some serpins occur in plants where their function is not known.

As used herein, the term “alpha 1-antitrypsin protein” denotes a glycoprotein. Alpha 1-antitrypsin is also referred to as alpha-1 proteinase inhibitor (A1PI) because it is a serine protease inhibitor (serpin), inhibiting a wide variety of proteases. It protects tissues from enzymes of inflammatory cells, especially elastase. An exemplary sequence for human Alpha 1-antitrypsin gene is deposited in the database Genbank under accession number NC008290.

As used herein, the term “an active fraction of” denotes a fraction of a protein with the activity of the complete protein”. For example, an active fraction of the trappin-2 protein denotes a fraction of the protein which conserves the capacity to inhibit the HNE or an active fraction of the Serpin family proteins denotes a fraction of the protein which conserves the capacity of inhibition.

As used herein, the term “food-grade bacterium” denotes a bacterium that is widely used in fermented foods and possesses a perfect safety profile recognized by the GRAS (Generally Recognized As Safe) and QPS (Qualified Presumption of Safety) status in USA and European Community, respectively. Such bacterium can be safely in functional foods or food additives with allegations concerning maintain in good health and well-being or prevention of disease.

As used herein, the term “probiotic bacterium” denotes a bacterium which ingested live in adequate quantities can exert beneficial effects on the human health. They are now widely used as a food additive for their health-promoting effects. Most of the probiotic bacteria are Lactic Acid Bacterium (LAB) and among them strains of the genera *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria.

As used herein, the term “*thyA* gene” denotes, the gene coding for thymidylate synthase which is an enzyme generating thymidine monophosphate (dTMP), which is subsequently phosphorylated to thymidine triphosphate used in DNA synthesis and repair.

As used herein, the term “Irritable Bowel Syndrome (IBS)” is a term for a variety of pathological conditions causing discomfort in the gastro-intestinal tract. It is a functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating, and alteration of bowel habits in the absence of any organic cause.

As used herein, the term “inflammatory bowel diseases (IBD)” is a group of inflammatory diseases of the colon and small intestine. The major types of IBD are Crohn's disease, ulcerative colitis and pouchitis.

Proteins and uses thereof:

A first object of the invention relates to a molecule selected from the trappin-2 protein or an active fraction of the trappin-2 protein, a member of the WAP family proteins or an active fraction of a member of the WAP family proteins, or a molecule selected from the serpin family or an active fraction of the serpin family for the treatment of Irritable Bowel Syndrome (IBS).

In a preferred embodiment, the member of the Serpin family is the alpha 1-antitrypsin protein.

In a preferred embodiment, said fraction of the protein comprises at least 75% identity over said protein, even more preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%.

Typically said protein or fraction of the protein thereof may be used in combination with an anti-inflammatory agent.

10 Proteins of the invention or fractions of the proteins thereof may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination(s).

15 Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce a relevant part of the said proteins or fraction of the protein, by standard techniques for production of proteins. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available protein synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions.

20 Alternatively, the proteins or fraction of the proteins of the invention thereof can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired polypeptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired protein or fraction of the protein, from which they can be later using well-known techniques.

25 Proteins or fraction of the proteins of the invention thereof can be used in an (e.g., purified) form or contained in a vector, such as a membrane or lipid vesicle (e.g. a liposome).

Food-grade bacteria

30

A further object of the invention relates to a recombinant food-grade bacterium comprising a gene selected from a gene coding for the trappin-2 protein or an active fraction of the trappin-2 protein, a gene coding for a member of the WAP family proteins or an active

fraction of a member of the WAP family proteins, or a gene coding for a member of the serpin family proteins or an active fraction of a member of the serpin family proteins.

5 In a preferred embodiment, the food-grade bacterium according to the invention is a probiotic bacterium.

In a preferred embodiment, the probiotic bacterium according to the invention comprises a defective auxotrophic gene, whereby survival of said bacterium is strictly dependent upon the presence of specific compounds.

10 In another preferred embodiment, the auxotrophic gene according to the invention is the *thyA* gene encoding the *thymidylate synthase*.

In another preferred embodiment, the auxotrophic gene according to the invention is the *alanine racemase (alr)* gene (Bron et al, 2002).

15 Inactivation of the *thyA* gene of the probiotic bacterium according to the invention renders it auxotrophic to thymidine which is absent from the gastrointestinal tract (GIT). This recombinant *thyA* mutant will be able to deliver its protein of interest but will not survive and thus persist in GIT limiting its dissemination and conferring the requested biological containment for recombinant bacteria. Similar results can be obtained with *alr* gene.

20 In another preferred embodiment, the selected gene is inserted in the *thyA* gene.

Preferably, the recombinant gene is located in the chromosome into the *thyA* gene locus which is thus inactivated by gene disruption. As used herein, the term "gene disruption" denotes disruption by insertion of a DNA fragment, disruption by deletion of the gene, or a part thereof, as well as exchange of the gene or a part thereof by another DNA fragment, and the disruption is induced by recombinant DNA techniques, and not by spontaneous mutation. Preferably, disruption is the exchange of the gene, or a part thereof, by another functional gene. Preferably, the defective recombinant *thyA* gene is a non-reverting mutant gene.

30 As used herein, the term "non-reverting mutant" denotes that the reversion frequency is lower than 10^{-8} , preferably the reversion frequency is lower than 10^{-10} , even more preferably, the reversion frequency is lower than 10^{-12} , even more preferably, the reversion

frequency is lower than 10^{-14} , most preferably, the reversion frequency is not detectable using the routine methods known to the person skilled in the art.

In a preferred embodiment, the gene according to the invention codes for the alpha 1-
5 antitrypsin protein, or another members of the Serpin family such as antiplasmin, alpha 1-
antichymotrypsin.

In a preferred embodiment, the food-grade bacterium strain according to the invention
is a *L. lactis* strain or a *Lactobacillus casei* strain or a *L. lactis htrA strain* [Poquet et al.,
10 2000] or a *Lactobacillus plantarum* strain or a *Bifidobacterium longum* strain.

In a preferred embodiment, the food-grade bacterium strain according to the invention
is a *Lactobacillus casei* strain.

15 In a most preferred embodiment, the gene according to the invention codes for trappin-
2.

Indeed, the inventors showed that trappin-2 is naturally expressed in the human
colonic mucosa, with a prominent expression in intestinal epithelial cells (Motta et al.) and
20 that, patients with IBD show a down-regulation of trappin-2 in tissues compared to healthy
subjects (Motta et al.).

Furthermore, the inventors have demonstrated in different models of colitis, that
trappin-2 overexpression is protective against the development of colitis (in constitutive and
transient expression). Moreover, trappin-2 overexpression in models of colitis is able to
25 completely inhibit the increase of elastase and trypsin-like activities associated with colitis.

Finally, trappin-2 overexpression in mice is also able to significantly inhibit colitis-
induced increases of pro-inflammatory cytokines and chemokines (IL-6, Il-17A, TNF-alpha,
Interferon-gamma, MCP-1 and KC).

All these results are in favour of delivery of trappin-2 and others proteases from the
30 WAP or Serpin family which have similar properties to treat IBD. As shown in the results
below, food-grade bacteria are the most safe and efficient means to deliver this type of
proteases to the gut.

In another preferred embodiment, the gene according to the invention codes for the alpha 1-antitrypsin protein.

Indeed, the alpha 1-antitrypsin protein inhibits trypsin-like activities associated with IBD (like colitis) and thus has similar effects as trappin-2.

5

In another preferred embodiment, the food-grade bacterium strain according to the invention is a *Lactobacillus casei* strain which comprises a gene coding for trappin-2.

10 In another preferred embodiment, the food-grade bacterium strain according to the invention is a *Lactobacillus casei* strain which comprises a gene coding for trappin-2 inserted in the *thyA* gene.

In another embodiment, the food-grade bacterium according to the invention is useful for the treatment of intestinal inflammatory conditions.

15 In another preferred embodiment, the food-grade bacterium according to the invention is useful for the treatment an IBD and/or IBS.

The inflammatory conditions may be selected from IBD, IBS, inflammatory pulmonary disease, inflammatory articular disease or inflammatory urogenital disease.

20

Compositions

Another object of the invention relates to a therapeutic composition comprising a food-grade bacterium according to the invention.

25

In a preferred embodiment, therapeutic composition according to the invention is intended for mucosal administration to a subject.

In another preferred administration, therapeutic composition according to the invention is intended for oral administration to a subject. For example, compositions can be in
30 the form of a suspension, tablet, pill, capsule, granulate or powder.

In a liquid therapeutic composition, the food-grade bacterium according to the invention is present, free and not immobilized, in suspension. The suspension has a

composition which ensures physiological conditions for a probiotic bacterium, so that in particular the osmotic pressure within the cell does not lead to lysis.

5 In a solid therapeutic composition, the food-grade bacterium according to the invention can be present in free, preferably lyophilized form, or in immobilized form. For example, the food-grade bacterium according to the invention can be enclosed in a gel matrix which provides protection for the cells.

10 A solid therapeutic composition intended for oral administration and containing the food-grade bacterium according to the invention in immobilized or non-immobilized form is preferably provided with a coating resistant to gastric juice. It is thereby ensured that the food-grade bacterium contained in the therapeutic composition can pass through the stomach unhindered and undamaged and the release of the food-grade bacterium first takes place in the upper intestinal regions.

15

In another aspect of the invention, the therapeutic composition contains sufficient colony-forming units (CFU) of the food-grade bacterium capable of forming the protein according to the invention so that with multiple administration of the therapeutic composition according to a patient, the state of the IBD or IBS is healed, the progression of the IBD or the IBS is stopped, and/or the symptoms of the IBD or IBS can be alleviated. According to the invention, it is in particular provided that a therapeutic composition contains 1×10^8 - 1×10^{11} , preferably 1×10^9 - 1×10^{10} CFU of the food-grade bacterium according to the invention.

20

In a further preferred embodiment of the invention, the therapeutic composition containing the food-grade bacterium is administered intrarectally. A rectal administration preferably takes place in the form of a suppository, enema or foam. Intrarectal administration is particularly suitable for chronic inflammatory intestinal diseases which affect the lower intestinal sections, for example the colon. Intranasal administrations are also suitable to treat chronic pulmonary diseases such as cystic fibrosis and BPCO.

25

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In another aspect, the invention relates to a food composition comprising a food-grade bacterium according to the invention.

In a preferred embodiment, food compositions according to the invention are intended for oral administration to a subject. For example, compositions can be in the form of a suspension, tablet, pill, capsule, granulate, powder or yogurt.

5 In a preferred embodiment, the food composition may contain 1×10^8 - 1×10^{11} , preferably 1×10^9 - 1×10^{10} CFU of the food-grade bacterium according to the invention.

In a preferred embodiment, the food composition may be administered to the patient at a daily dose of 10^{10} bacteria.

10

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

15

FIGURES:

20 Figure 1: Weight differences (A), macroscopic score (B), Wall thickness (C), and myeloperoxidase (MPO) activity (D) in colonic tissues from mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *L. lactis*, or recombinant *L. lactis* strains expressing either Elafin or IL-10. Significant differences compared to PBS-treated mice that have received DSS were noted * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.005$. Significant differences compared to wild-type *L. lactis*-treated mice were noted ψ for $p < 0.05$, $\psi \psi$ for $p < 0.01$, and $\psi \psi \psi$ for $p < 0.05$. Significant differences between PBS + DSS group and the PBS-water group were noted # for $p < 0.05$, ## for $p < 0.01$, and ### for $p < 0.005$. Significant differences compared to the IL-10 recombinant *L. lactis* group were noted ω for $p < 0.05$, $\omega \omega$ for $p < 0.01$, and $\omega \omega \omega$ for $p < 0.005$.

25

30

Figure 2: Trypsin-like activity (A) and elastase activity (B) in washes of the colonic lumen of mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *L. lactis*, or recombinant *L. lactis* strains expressing either Elafin or IL-10. ε means undetectable levels. Significant differences

compared to PBS-treated mice that have received DSS were noted * for $p < 0.05$, ** for $p < 0.01$. Significant differences compared to wild-type *L. lactis*-treated mice were noted ψ for $p < 0.05$. Significant differences between PBS + DSS group and the PBS-water group were noted # for $p < 0.05$, ## for $p < 0.01$, and ### for $p < 0.005$.

5

Figure 3: Macroscopic score (A), Wall thickness (B), and myeloperoxidase (MPO) activity (C) in colonic tissues from mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *Lb. casei*, or recombinant *Lb. casei* strains expressing Elafin. Significant differences compared to PBS-treated mice that have received DSS were noted * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.005$. Significant differences compared to wild-type *Lb. casei*-treated mice were noted ψ for $p < 0.05$, $\psi \psi$ for $p < 0.01$, and $\psi \psi \psi$ for $p < 0.005$.

10

Figure 4: Trypsin-like activity (A) and elastase activity (B) in washes of the colonic lumen of mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *Lb. casei*, or recombinant *Lb. casei* expressing elafin. Significant differences compared to PBS-treated mice that have received DSS were noted * for $p < 0.05$, and significant differences compared to wild-type *Lb. casei*-treated mice were noted ψ for $p < 0.05$, and $\psi \psi$ for $p < 0.01$.

15

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Figure 5: Protein concentration of RANTES (A), TNF α (B), IL-6 (C), MCP-1 (D), KC (E), INF γ (F) and IL-17 (G) detected in colonic tissues from mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *Lb. casei*, or recombinant *Lb. casei* expressing Elafin. ϵ means undetectable levels. Significant differences compared to PBS-treated mice that have received DSS were noted * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.005$. ψ showed significant differences for $p < 0.05$, compared to mice treated with wild-type *L. lactis*. Significant differences between PBS + DSS group and the PBS-water group were noted # for $p < 0.05$, ## for $p < 0.01$, and ### for $p < 0.005$.

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Figure 6: Protein concentration of IL-2 (A), IL-4 (B), IL-5 (C), IL-10 (D) and IL-13 (E) detected in colonic tissues from mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with wild-type *Lb. casei*, or recombinant *Lb. casei* expressing Elafin. Significant differences compared to PBS-treated

mice that have received DSS were noted * for $p < 0.05$, and ** for $p < 0.01$. Significant differences compared to mice treated with wild-type *Lb. casei* were noted ψ for $p < 0.05$, and ψ for $p < 0.01$.

5 Figure 7: Total number of pain behaviors (A) or number of abdominal contractions and licking, stretching and squashing behaviors (B) in mice that have received intracolonicly PBS (n=5), or Mustard oil (0.01% (v/v) in ethanol 70%), and that have received in for the previous 7-days, pre-treatments by oral gavage of PBS (n=8), wild-type *L. lactis* (n=8), or recombinant *L. lactis* expressing either elafin (n=8) or IL-10 (n=5). Significant
10 differences compared to PBS-treated mice that have received mustard oil were noted * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.005$. ψ showed significant differences for $p < 0.05$, compared to mice treated with wild-type *L. lactis*.

Figure 8: Elafin secreted by *L. lactis* wt and *htrA* strains. Western blot experiments
15 performed with antibodies anti-elafin on cellular (C) and supernatant (S) extracts of wild type (*wt*) or *htrA* (*htrA*) strains. Elafin production was induced by nisin from exponential-phase cultures of *wt* or *htrA* strains (both containing the expression vector where elafin gene expression can be induced by nisin addition).

20 Figure 9: Protective effects of *L. lactis* wt and *htrA* mutant strains in DSS 5% -induced colitis model. Macroscopic (A), histological damages (B) and MPO activities (C) were evaluated in different groups of 10 mice treated either with water (negative control) or with DSS 5%. Two first control groups were treated i) with water and orally fed with PBS (negative control group) and ii) with DSS 5% and orally fed with PBS (positive control group). The other groups were all treated with DSS 5% and with either wt strain (WT), wt strain expressing elafin (Elafin) and *htrA* mutant strain expressing elafin (Elafin+).
25

Figure 10: Protective effects of *L. casei* wt strain and SOD-expressing, elafin-expressing and IL-10-expressing *L. casei* strains in DSS 5% -induced colitis model.
30 Macroscopic (A), histological damages (B) and MPO activities (C) were evaluated in different groups of 10 mice treated either with water (negative control) or with DSS 5%. Two first control groups were treated i) with water and orally fed with PBS (negative control group) and ii) with DSS 5% and orally fed with PBS (positive control group). The other groups were all treated with DSS 5% and with either *L. casei* wt strain (WT) or *L. casei*

strains expressing superoxide dismutase (SOD), elafin (Elafin) or IL-10. * and ** indicate that the data are significantly different ($P < 0.05$) from the data obtained with *L. casei wt*.

Figure 11: Myeloperoxydase (MPO) activity (D) in colonic tissues from mice.
 5 Myeloperoxydase (MPO) activity (D) was measured in colonic tissues from mice that had water or water + DSS (3%) in their drinking bottles, and that received daily oral treatments for 7-days with i) WT *L. lactis*, recombinant *L. lactis* and *L. lactis htrA* strains expressing either Elafin or IL-10 and with ii) *L. casei wt* strain and SOD-expressing, elafin-expressing and IL-10-expressing *L. casei* strains. * and ** indicate that the data are significantly different
 10 ($P < 0.05$) from the data obtained with *L. casei wt*.

Figure 12: Elafin secretion in a *L. lactis* WT strain where the elafin gene is expressed under the control of an EDTA-inducible promoter [Lull D and Poquet I. 2004; EP 1 537 215 and FR 98 16462]. The *L. lactis* WT strain expressing elafin was grown overnight in the
 15 presence (+) or not (-) of EDTA, a chelator agent (in this construct, elafin gene expression is controlled by a lactococcal promoter that can be induced by EDTA addition: on the chromosome, this promoter controls the expression of genes encoding an ABC uptake system specific for zinc and is derepressed under zinc starvation conditions that can be mimicked by EDTA addition). Proteins were then extracted and fractionated between cell (C) and
 20 supernatant fractions (S) and Western blot experiments were performed using antibodies anti-elafin.

EXAMPLE:

25

Material & Methods

Cloning of Elafin in recombinant lactic acid bacteria (*Lactococcus lactis* and
Lactobacillus casei)

30

Cloning and expression of elafin in lactic acid bacteria

Gene coding for elafin was PCR amplified from plasmid DK6-elafin(14). Sequences of primers used were: 5' forward Elafin (CCAATGCATCAGCAGCTGTCACGGG
 AGTTCC) (S E Q I D N ° 1) and 3' reverse-Elafin

(GGACTAGTCCTCACTGGGGAACGAAACA GGCC) (SEQ ID N°2). Primers were designed to eliminate first codons of elafin region encoding for signal peptide (SP) and was replaced by the SP of Usp45 protein (PS_{Usp45}), the main secreted protein from *L. lactis*. To that aim, PCR product was digested, purified, and cloned in pSEC, a *L. lactis* secretion vector.

5 In the resulting plasmid pSEC:elafin, elafin is fused in frame with a DNA fragment encoding for RBS and PS_{Usp45}. Expression of the cassette is controlled by the inducible promoter P_{nisA}, the activity of which depends upon the concentration of nisin used. This plasmid was then introduced in a *L. lactis* strain bearing the nisin regulatory genes *nisR* et *nisK* (*L. lactis* NZ9000) to give rise the recombinant strain: NZ(pSEC :elafin). The tools used (replicons,

10 promoter, RBS and SP) are functional in lactobacilli strains such as *Lactobacillus casei* and *Lb. plantarum*. These two strains (each bearing the genes *nisRK* on their chromosome) have been chosen because of their persistence ability in the digestive tract (up to 4 days, as opposed to 24 to 48 hr in *L. lactis*). In addition, we have demonstrated recently that the *Lb. casei* BL23 strain possesses anti-inflammatory properties in a DSS-induced colitis model [Rochat et al,

15 2007]. We therefore have at our disposal non-immuno-modulatory strains weakly (*L. lactis*) and strongly (*Lb. plantarum*) persistent as well as immuno-modulatory strains strongly persistent (*Lb. casei*), allowing us to evaluate the feasibility of combining the intrinsic anti-inflammatory effects of the strains used with that of the molecules over-expressed.

20 For the induction of the P_{nisA} promoter, *L. lactis* recombinant strains were cultured to OD₆₀₀~0.4-0.6 and then induced with 10 ng/ml de nisin (Sigma) during 1 h. Functionality of this induction was then tested as follows: NZ (pSEC:elafin) cultures (final OD₆₀₀~1) were separated into pellets and supernatants and content in elafin was measured by ELISA and/or Western Blot.

25

Animals

C57Bl6 mice (6-8 weeks old) were obtained from Janvier (St Quentin Fallavier) and were kept at room temperature, under 12h light/dark cycles and having free access to food and water, except the day before the induction of colitis, where they were fasted for 12h. All

30 procedures were approved by Institutional animal care committee and veterinary services.

Induction of colitis and study design

Colonic inflammation was induced by treatments with Dextran Sodium Sulfate (DSS). In details, DSS was dissolved in drinking water (3 or 5% wt/vol) and the animals were free to

drink this solution for 7-days. Water consumption was measured in the DSS-treated groups and compared to groups of naïve mice drinking water: no difference was observed for the volume of liquid consumed, between water and DSS-drinking mice. Mice were treated daily orally, with 100 µl of $5 \cdot 10^9$ colony forming units (cfu) of wild-type, elafin-recombinant *L. lactis* or *Lb. casei*, or bacterial medium alone. The first treatment started at the same time DSS was added to drinking water and the last treatment was on the day of the sacrifice (day 7). Body weight and survival rate were measured daily after the induction of colitis. On day 7 after adding DSS to their drinking water, mice were sacrificed and colons were harvested for measure of several parameters of inflammation: macroscopic score, bowel thickness, myeloperoxidase (MPO) activity, proteolytic activity, cytokine expression.

Measure of inflammatory parameters

Macroscopic damage was evaluated as previously described (5'15'16). Briefly, when observed, the following parameters were given a score of 1: haemorrhage, edema, stricture, ulceration, fecal blood, mucus, and diarrhoea. Erythema was scored a maximum of 2 depending on the length of the area being affected (0: absent, 1: less than 1 cm, 2: more than 1 cm). Adhesion was scored based on its severity (0: absent, 1: moderate, 2: severe).

MPO was measured as an index of granulocyte infiltration as previously described (5'15'16), in colonic tissues harvested at the time of the sacrifice. Tissue samples were homogenized in a solution of 0.5 % hexadecyltrimethylammonium bromide in phosphate buffer (pH 6), and centrifuged at 13 000 X G for 2 min. Supernatants were added to a buffer containing 1 % hydrogen peroxide and O-dianisidine dihydrochloride. Optical density readings for the enzymatic solution were read for 2 min at 450 nm.

For cytokine and chemokine protein measures, frozen colonic samples harvested at sacrifice were homogenized using a polytron for 30 s at 4°C in 500µl of cell lysis buffer (20mM Tris-Hcl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin; Cell Signalling, Sigma) supplemented with anti-proteases (Roche Diagnostics, Meylan, France) cocktail. After centrifugation (10 000Xg, 10 min, 4°C), supernatants were filtered on QIAshredder columns (Qiagen, France) and fifty microliters of this homogenate was used for simultaneous dosage of cytokines and chemokines using cytometric bead array on fluorescent cell sorter FACSCalibur. Raw values were normalized to tissue weight (average from 30 to 50 mg) and cytokine concentrations were extrapolated from standard

curves with the help of FCAP Array® software. In accordance with the manufacturer's information, only values above the limit of cytokine detection were considered.

Serine protease activity in colonic tissues and luminal washes

5 As previously described (17), upon sacrifice, the entire colon was excised and 1ml PBS was instilled and washed twice through the lumen. Proteolytic activities (trypsin-like and elastase activity) were measured both in those luminal washes. Trypsin-like and elastase-like activities were measured using tosyl-Gly-Pro-Arg-*p*-nitroanilide (150 µM, Sigma) and MeO-succinyl-Ala-Ala-Pro-Val- *p*-nitroanilide (100 µM, Sigma, Saint Quentin Fallavier, France) respectively as substrates. Samples (20 µl for trypsin activity or 10 µl for elastase activity) 10 were re-suspended in their respective buffer : 100mM Tris/HCl, 1mM CaCl₂, pH=8 for trypsin activity and 50 mM Tris-HCl, 500 mM NaCl, 0.1% Triton X100 for elastase activity. The change in absorbance at 405 nm was determined over 30 minutes at 37°C with a microplate reader NOVOstar™ (BMG Labtech, France). Activity was compared to known 15 standard dilution of trypsin from porcine pancreas (Sigma) or human neutrophil elastase (Sigma). Protein concentration in the luminal washes was determined using colorimetric dosage of bicinchoninic acid on microplate (BCA kit®, Pierce, Thermo Scientific, Courtaboeuf, France) and was used to standardize the proteolytic activity in each samples.

20 *Induction and measure of visceral pain behaviors in response to Mustard oil*

Cultures of the *L. lactis* three strains of (*L. lactis* wt, *L. lactis*-Elafin, *L. lactis*-IL-10) were performed in M17 medium (Oxoid) supplemented with glucose (0.5%) supplemented with Chloramphenicol (10 µg/mL) at 30°C without shaking. Bacteria from overnight cultures were grown in fresh medium at 1/50 (v/v) until OD₆₀₀=0.4 to 0.6. Bacteria were then cultured 25 for one more hr with Nisin (1 ng/mL), added to enable recombinant protein expression. Bacteria were harvested by centrifugation at 450 g and washed with sterile PBS. The pellets were resuspended in sterile PBS at a final concentration of 5X10¹⁰ cfu/mL. Groups of 4 to 8 mice were treated daily with 100µL (5X10⁹ cfu) of bacterial suspension by intragastric administration for seven days. At day 8, mice were administered with 50 µL of PBS or 30 mustard oil (0.01% (v/v) in ethanol 70%) by intracolonic instillation, performed under slight isoflurane anesthesia. The number of pain-related behavioral responses (abdominal retractions, licking of the abdomen, stretching, and squashing of the lower abdomen against the floor) were then counted for 20 min.

Statistics

Comparisons among groups were made using a 2-tailed Student's t test with Bonferroni correction. Data are expressed as mean \pm SEM, and a P value less than 0.05 was considered significant.

5

Results

Recombinant Lactococcus lactis expressing elafin protects against the development of DSS colitis in mice

As expected, DSS-induced colitis (5% DSS) caused severe weight loss in all groups of mice compared to control mice that drank water. None of the lactic acid bacteria treatments significantly modified this weight loss (Fig. 1A). DSS in drinking water also caused macroscopic damage, increased wall thickness and increased MPO activity in colonic tissues (Fig. 1 B, C, D). Mice that were treated with wild-type *L. lactis* did not show significant decrease in colonic wall thickness and MPO activity, only a slight decrease in macroscopic damage score was observed in that group, compared to DSS alone-treated mice. In contrast, mice treated with recombinant *L. lactis* expressing elafin showed after the induction of DSS colitis a significantly reduced macroscopic damage score and significant less increase in colonic wall thickness, but MPO activity was not different from DSS alone group (Fig. 1B, C, D). In addition, mice treated with recombinant *L. lactis* expressing IL-10 cytokine showed reduced macroscopic damage score and MPO activity after the induction of DSS colitis, but wall thickness was not modified by this treatment compared to DSS alone (Fig. 1B, C, D). In non-inflamed mice, none of the treatments modified the inflammatory parameters compared to naïve control mice.

DSS-induced increase in trypsin-like activity was significantly reduced in mice treated with recombinant *L. lactis* expressing Elafin, but was not changed in mice treated with wild-type *L. lactis* or recombinant *L. lactis* expressing IL-10 (Fig. 2A). Only treatment with recombinant *L. lactis* expressing elafin was able to significantly reduce DSS-induced increase in elastase activity (Fig. 2B).

30

Recombinant Lactobacillus casei expressing either elafin protects against the development of DSS colitis in mice

While mouse treatment with wild-type *Lb. casei* significantly reduced the macroscopic scores observed after the induction of DSS colitis, this treatment failed to reduce the increased wall thickness and MPO activity compared to DSS alone (Fig. 3 A, B, C). In contrast, treatments with recombinant *Lb. casei* expressing elafin significantly reduced all parameters of inflammation: macroscopic damage score, colonic wall thickness and MPO activity (Fig. 3A, B, C).

DSS-induced increase in trypsin-like activity was significantly reduced in mice treated with recombinant *Lb. casei* expressing Elafin, compared to mice treated with wild-type *Lb. casei* (Fig. 4A). The level of elastase activity was also significantly reduced in mice with colitis (DSS) treated with recombinant *Lb. casei* expressing elafin, compared to inflamed (colitis) mice treated with wild-type *Lb. casei*, or even compared to inflamed mice treated with PBS (Fig. 4B).

Protein expression of the chemokine RANTES was not significantly increased by DSS colitis at the observed time-point (7-days after the start of DSS treatment). Wild-type or Elafin-secreting *Lb. casei* failed to modify the level of RANTES expression in DSS-treated mice (Fig. 5 A). TNF α , IL-6, MCP1, KC, INF γ and IL-17A were all significantly increased by DSS colitis, 7 days after its induction (Fig. 5B to G). Treatment of mice with recombinant *Lb. casei* expressing elafin significantly reduced protein expression of IL-6, MCP1, KC and IL-17 (Fig. 5C, D, E, G), but failed to reduce the level of expression of other pro-inflammatory cytokines such as TNF α and INF γ (Fig. 5B and F). Interestingly, while DSS colitis did not cause any increase in the cytokines IL-2, IL-4, IL-5, IL-10 and IL-13 (Fig. 6 A to E), treatment of mice with *Lb. casei* recombinant for elafin raised significantly the expression of those cytokines, but only in a colitis context (after DSS treatment). This increase in Th2 cytokines in response to *Lb. casei* recombinant for elafin, could explain, at least in part, the anti-inflammatory effects of this recombinant bacteria. Levels of IL2, IL-4, IL-5, IL-10 and IL-13 were not modified by any of the other treatments in either inflamed (DSS) or non-inflamed mice (Fig. 6 A to E).

Recombinant Lactococcus lactis for the expression of elafin decrease visceral pain behavior

Intracolonic administration of mustard oil caused a significant increase in the number of pain behaviors: both the number of abdominal retractions and the number of integrated pain behaviors such as licking, stretching, and squashing against the floor (Fig. 7 A and B). Considering all behaviors together, treatment with wild-type, IL-10 recombinant or elafin-

secreting *L. lactis* had no effect (Fig. 7A). When considering only the integrated pain behaviors, elafin-, but not IL-10- recombinant *L. lactis* or wild-type significantly reduced the number of pain behaviors (Fig. 7B). Moreover, only recombinant *L. lactis* expressing elafin induced a notable decrease in the number of pain behaviors compared both to PBS treatment or wild-type *L. lactis* treatment (Fig. 7B).

Results with the htrA strain

L. lactis expresses only one housekeeping extracellular protease called *htrA* which degrades all the unfolded exported proteins (Poquet et al, 2000 and patent applications US 6,994,997 and FR2787810). A *L. lactis* mutant strain inactivated in *htrA* gene was constructed and allowed increasing the production rate of several heterologous secreted proteins in *L. lactis* (Poquet et al, 2000 and Miyoshi et al, 2002). According to the invention, the elafin expression cassette was cloned in the *htrA* mutant. Elafin production levels in the *htrA* mutant and in the wild type (wt) strain were compared by Western blot experiments (Fig. 8). A significant increase of secreted elafin was observed in the supernatant of the *htrA* mutant compared to the wt strain. Thus, the *htrA* strain will allow higher production and secretion levels of elafin.

These two strains were then tested in DSS-induced colitis model and we confirmed *in vivo* that the *htrA* mutant protects the mice against the colitis damages better than the wt strain (Fig. 9 A, B and C).

Comparative results

The protective effects of IL-10, superoxide dismutase (SOD) and elafin-producing *L. casei* strains were evaluated in parallel in DSS 5%-induced colitis model. It should be reminded that *L. casei* possesses two major differences compared to *L. lactis*: i) higher persistence in the GIT and ii) intrinsic anti-inflammatory properties (Rochat et al, 2007; Watterlot et al, 2010). As shown in Fig.10A/B/C, the best protective effects on the three criteria (macroscopic, histological and MPO activity) are obtained with Elafin-producing *L. casei* strain followed by SOD-producing *L. casei* strain. *L. casei* strain producing IL-10 provided only poor effects.

Moreover, elafin-producing *L. casei* strain afforded a better protection than the two *L. lactis* strains (wt *L. Lactis* and *htrA* strain) (Fig. 11).

These results are most surprising considering the fact that elafin possesses in vitro and in vivo antibacterial activities [Simpson AJ et al., 1999]. Accordingly, the skilled person would have expected a poor or no production at all by the bacterium host. On the contrary, the results obtained by the inventors show a very good production of elafin by the probiotic and hence a therapeutic effect.

Moreover, both in vitro and in vivo studies (including clinical studies) showed that the lack of the host antimicrobial shield is potentially deleterious in colonic diseases [Salzman NH et al., 2003 and Bevins CL et al., 2009].

Thus, the pleiotropic anti-microbial/anti-inflammatory activity of elafin makes it a very good therapeutic molecule candidate as compared to IL-10.

EDTA induction of Promoter Zinc (PZn) zitR-controlled expression in L. lactis.

Production of elafin in *L. lactis* driven by PZn *zitR* [Llull D and Poquet I. 2004] was tested by Western blot analysis after 1 h of induction with 1 mM of EDTA. Non-induced cultures samples, cellular pellet (C) and supernatant (S), produce very low levels and secretion of elafin while induced cultures result in higher levels of expression and secretion (fig. 12).

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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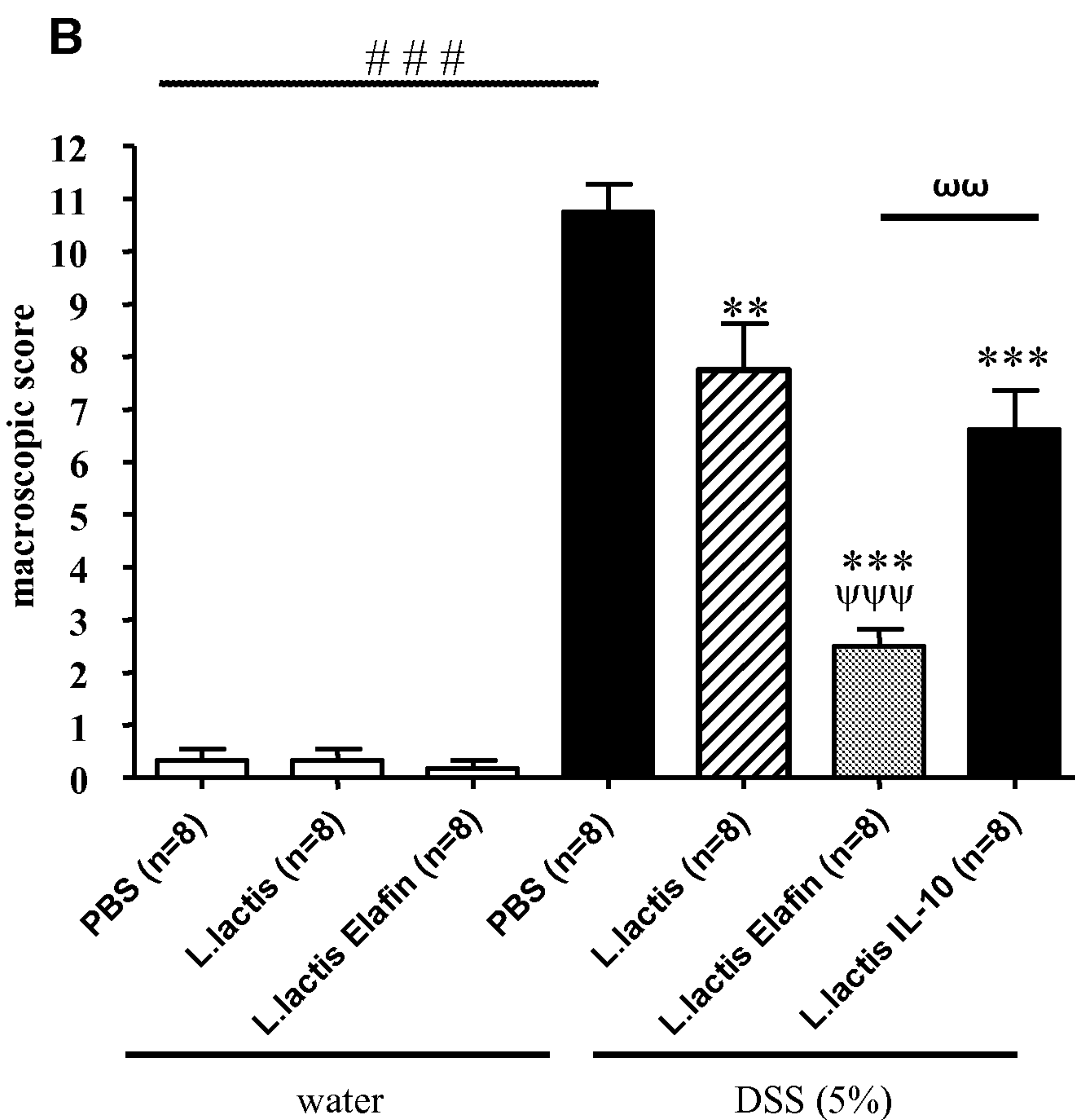
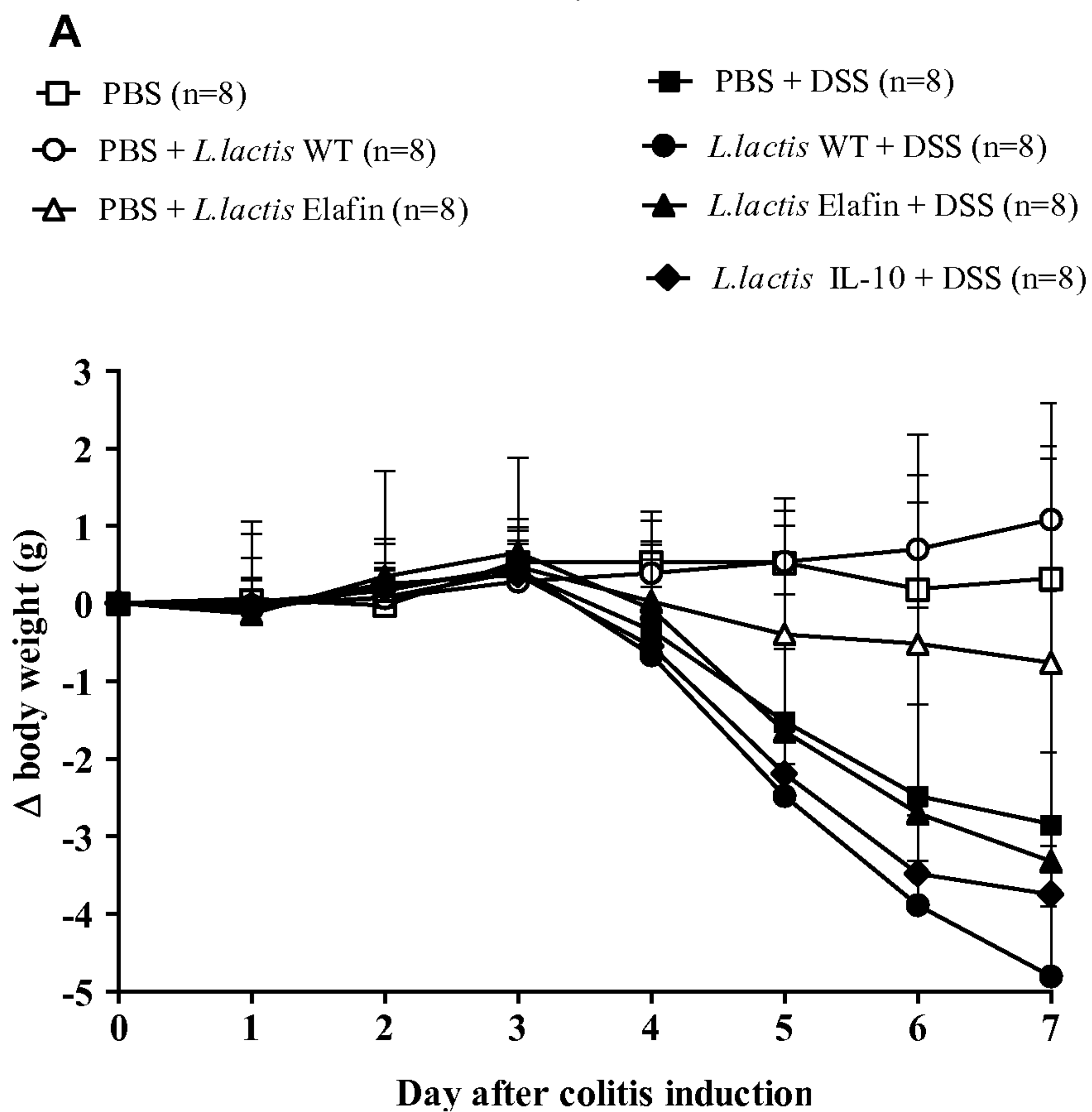
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CLAIMS:

1. A molecule selected from the trappin-2 protein or an active fraction of the trappin-2 protein, a member of the WAP family proteins or an active fraction of a member of
5 the WAP family proteins or a member of the Serpin family or an active fraction of a member of the Serpin family for the treatment of Irritable Bowel Syndrome (IBS).
2. A molecule according to the claim 1 expressed by a host cell genetically engineered.
3. A recombinant food-grade bacterium comprising a gene selected from a gene coding
10 for the trappin-2 protein or an active fraction of the trappin-2 protein, a gene coding for a member of the WAP family proteins or an active fraction of a member of the WAP family proteins or a gene coding for a member of the serpin family proteins or an active fraction of a member of the serpin family proteins.
4. A bacterium according to claim 3 wherein the food-grade bacterium is a probiotic bacterium.
- 15 5. A probiotic bacterium according to claim 3 or 4 wherein the bacterium comprises a defective auxotrophic gene, whereby survival of said bacterium is strictly dependent upon the presence of specific compounds.
6. A probiotic bacterium according to claim 5 wherein the defective auxotrophic gene is the thyA gene.
- 20 7. A probiotic bacterium according to claim 4 wherein the selected gene is inserting in the thyA gene.
8. A probiotic bacterium according to claim 3 to 5 wherein the gene coding for the WAP or Serpin family progene is trappin-2 or the alpha 1-antitrypsin protein.
9. A food-grade bacterium according to anyone of claim 3 to 6 selected from
25 Lactococcus lactis, Lactobacillus casei, Lactobacillus plantarum.
10. A food-grade bacterium according to anyone claims 3 to 9 for the treatment of an inflammatory condition.

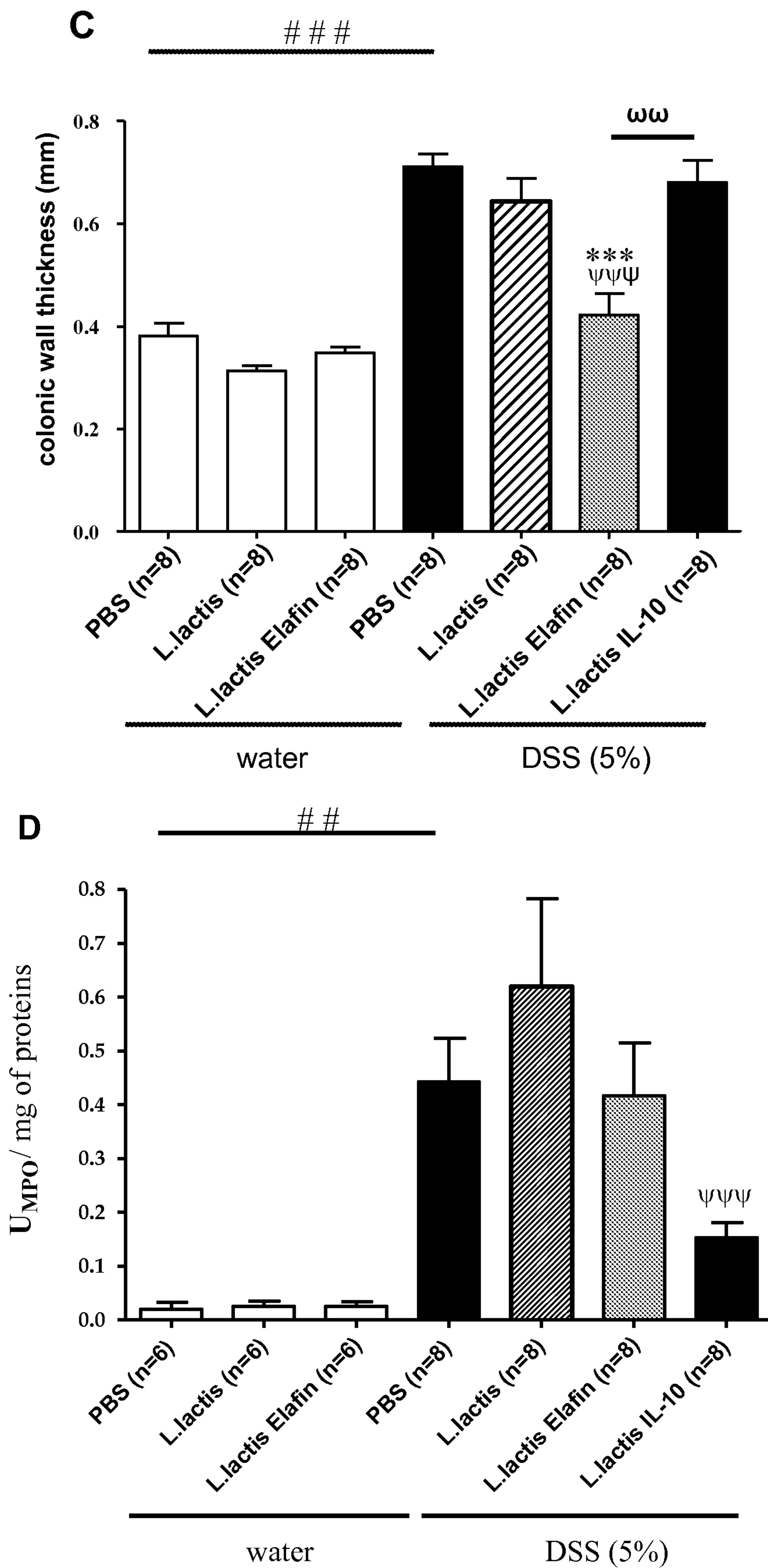
11. A food-grade bacterium according to claim 10 wherein the inflammatory condition is selected from Inflammatory Bowel Disease, Irritable Bowel Syndrome, inflammatory pulmonary disease, inflammatory articular disease or inflammatory urogenital disease.
12. A therapeutic composition comprising a food-grade bacterium according to anyone of
5 claims 3 to 9.
13. A food composition comprising a food-grade bacterium according to anyone of claims 3 to 9.
14. A composition according to any one of claims 10 and 11 wherein the composition is intended for oral administration to a subject.

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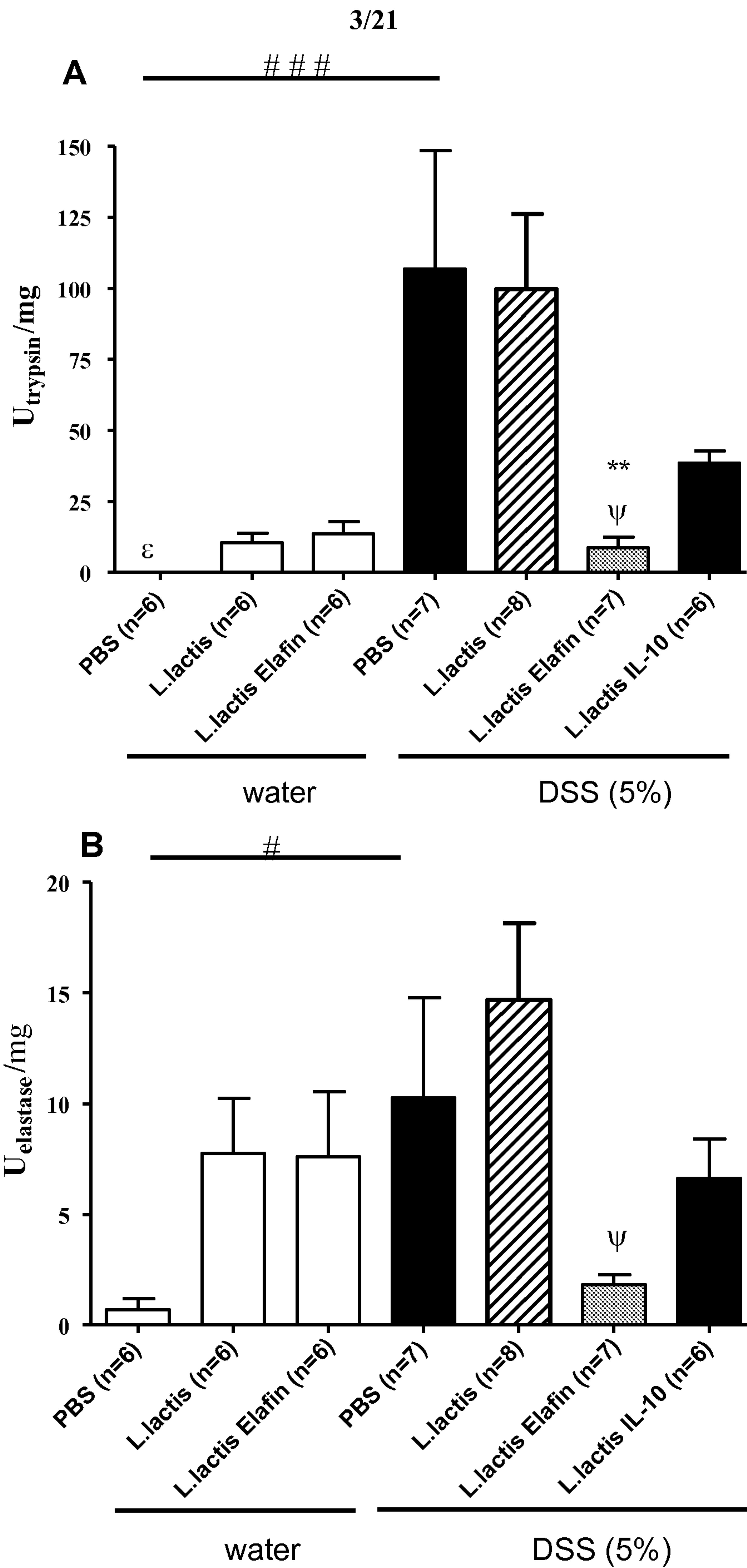


Figures 1 A and B

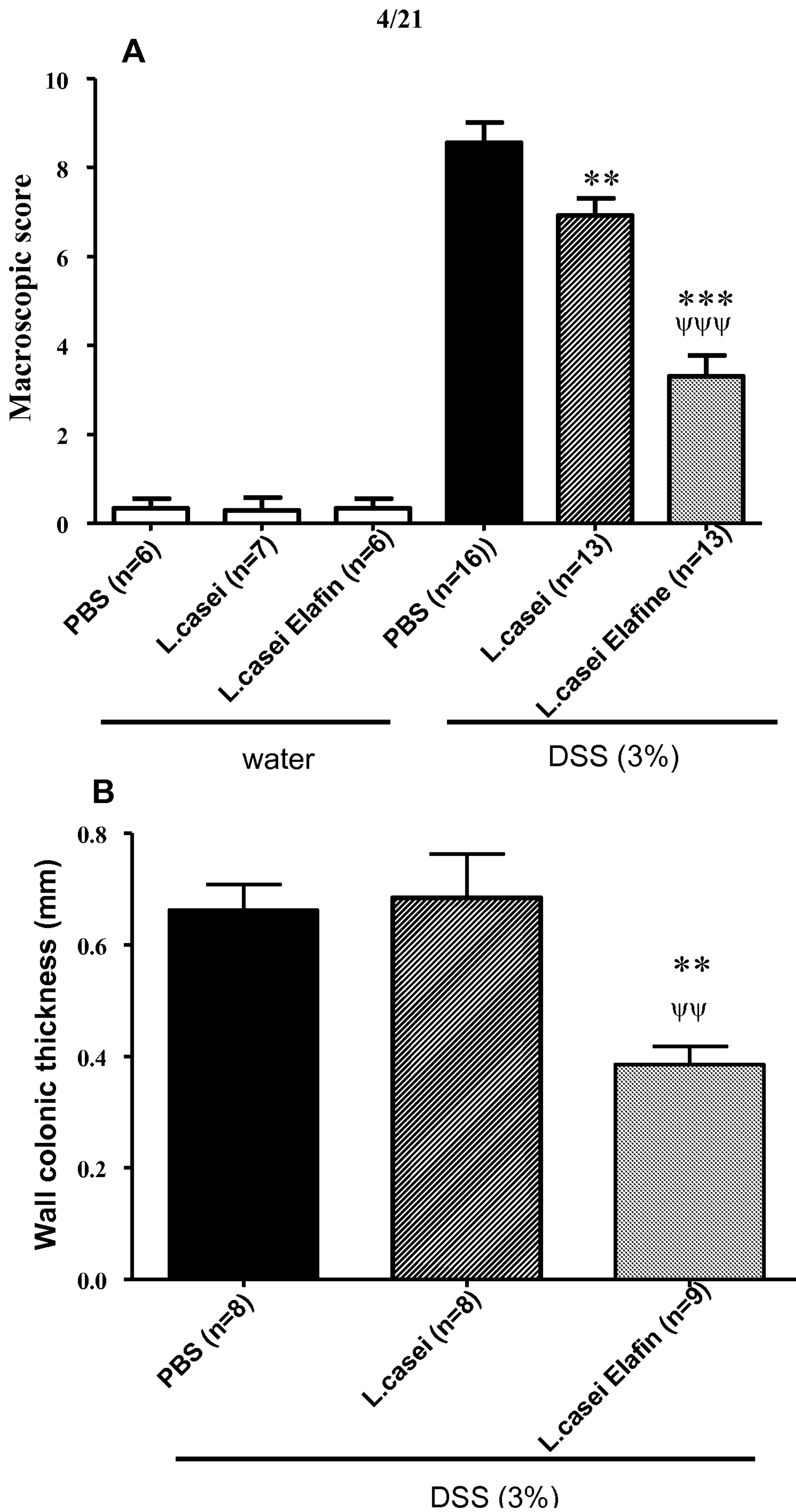
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Figures 1 C and D



Figures 2 A and B



Figures 3 A and B

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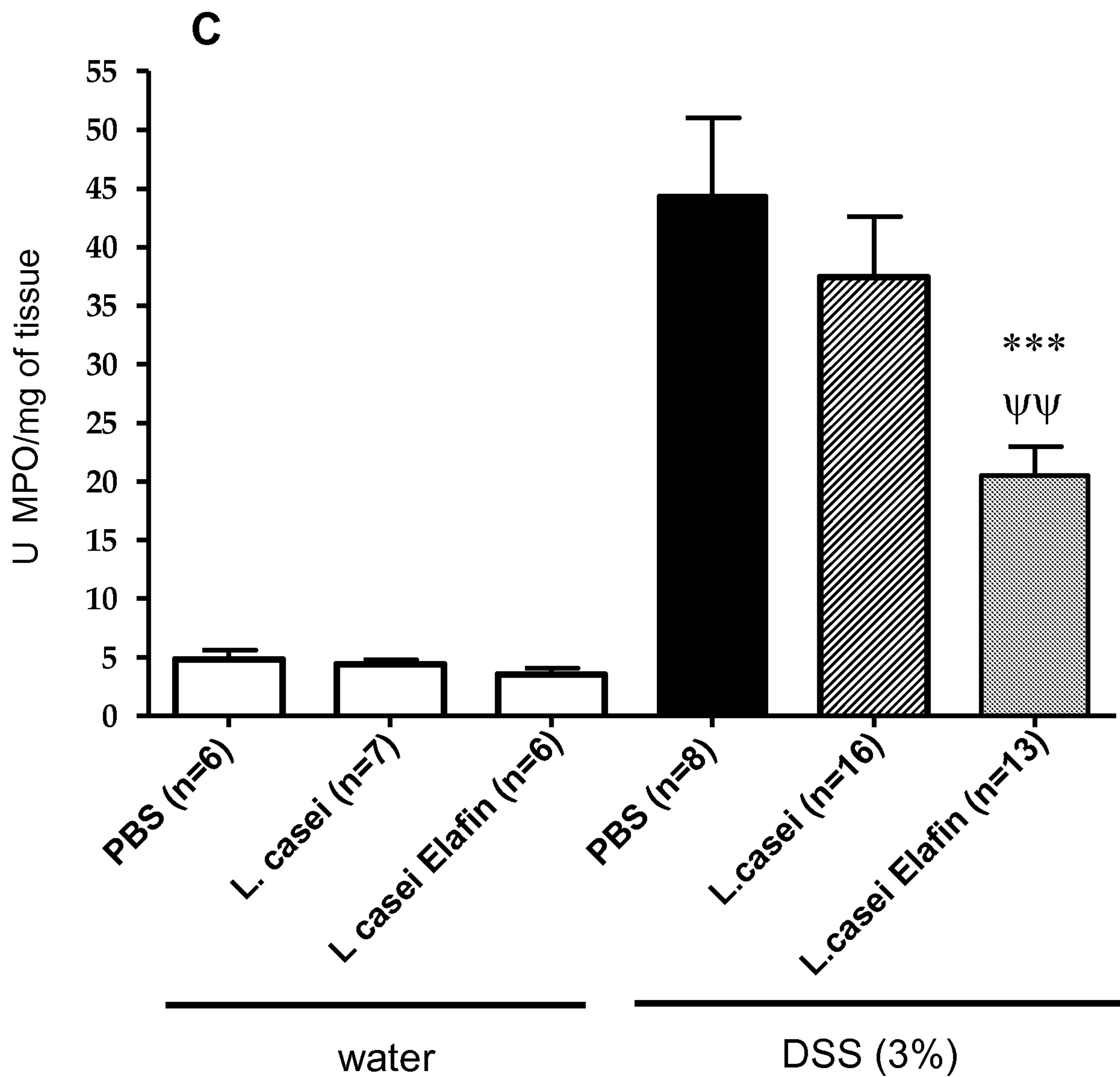
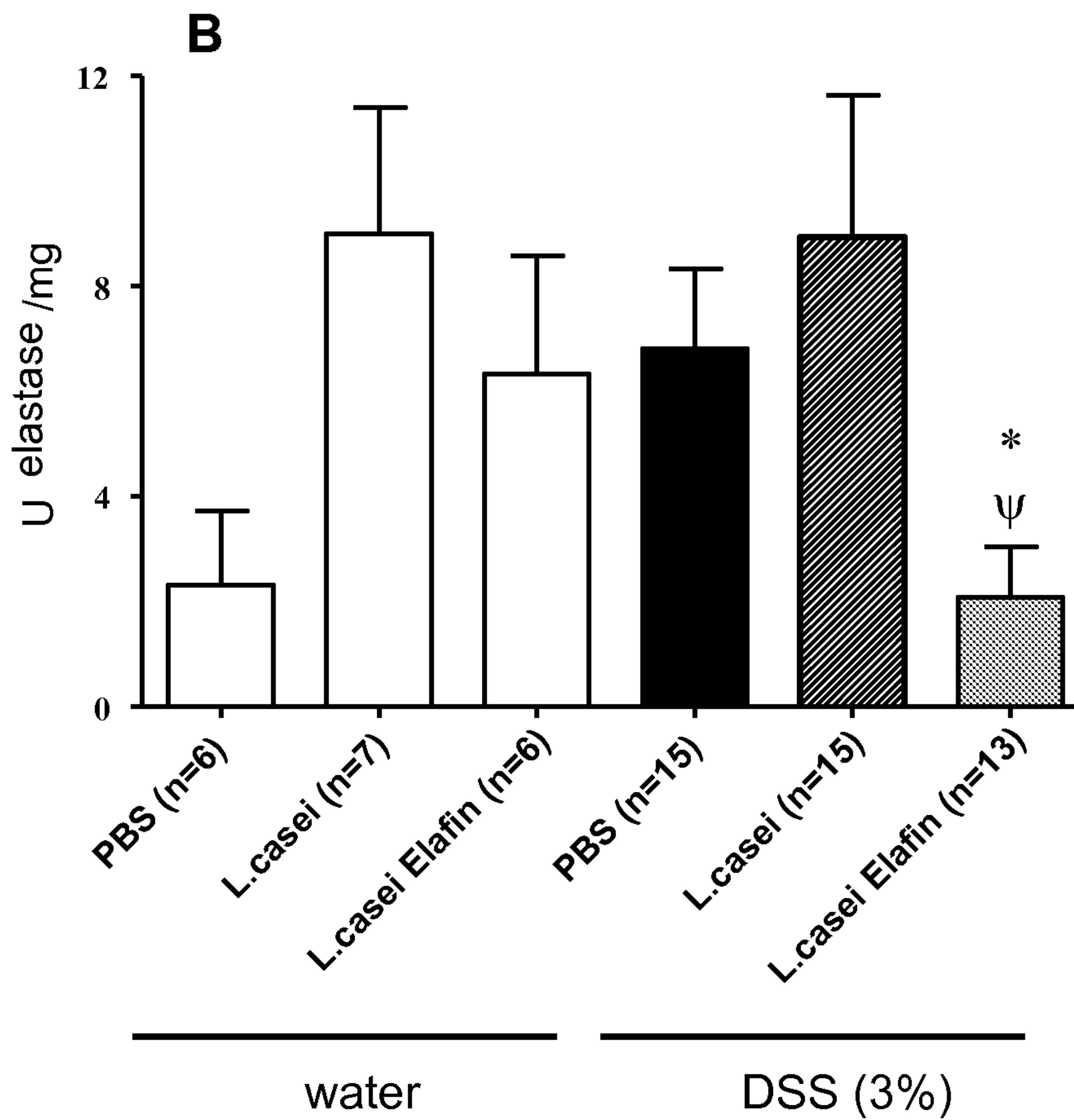
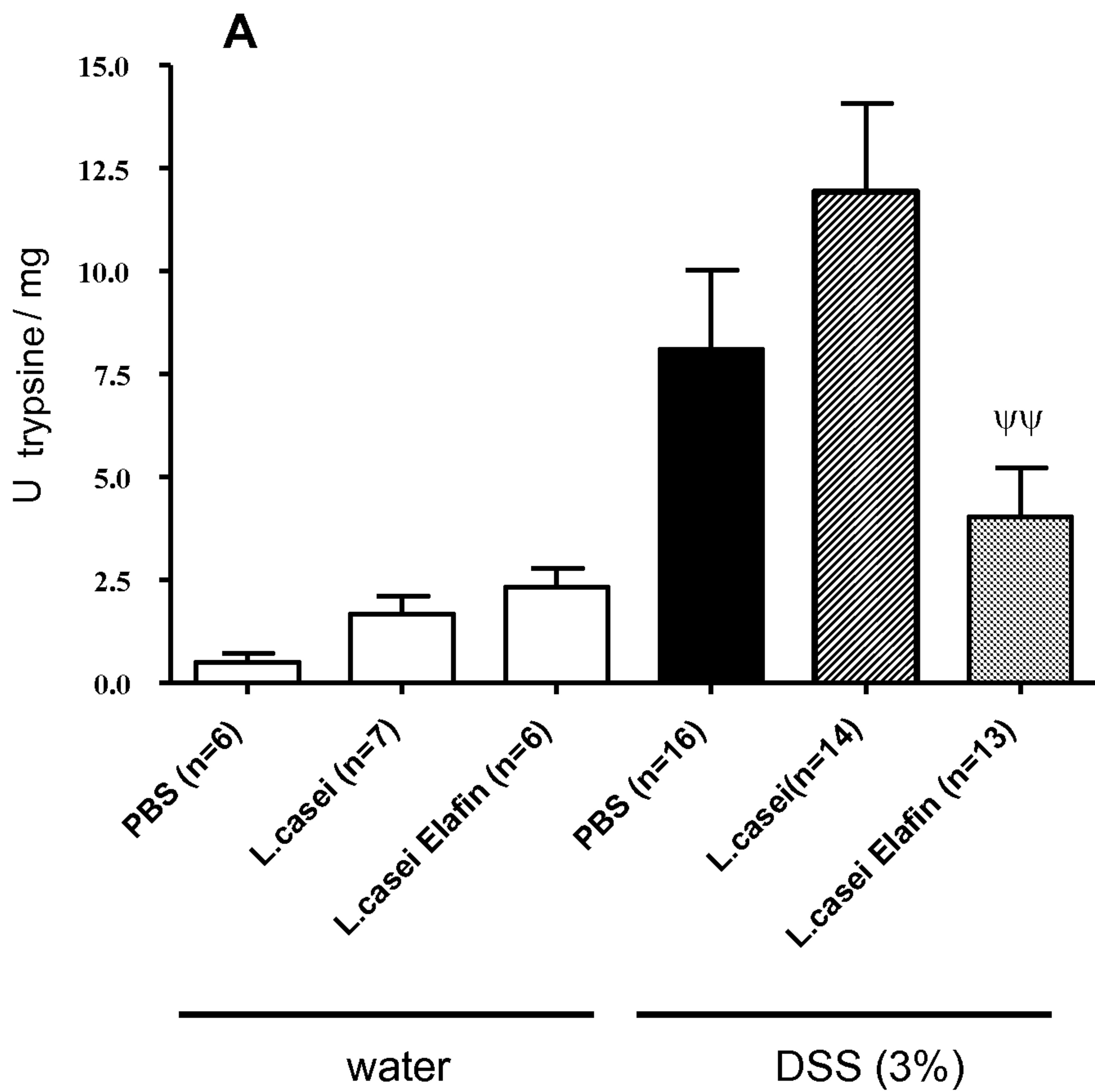


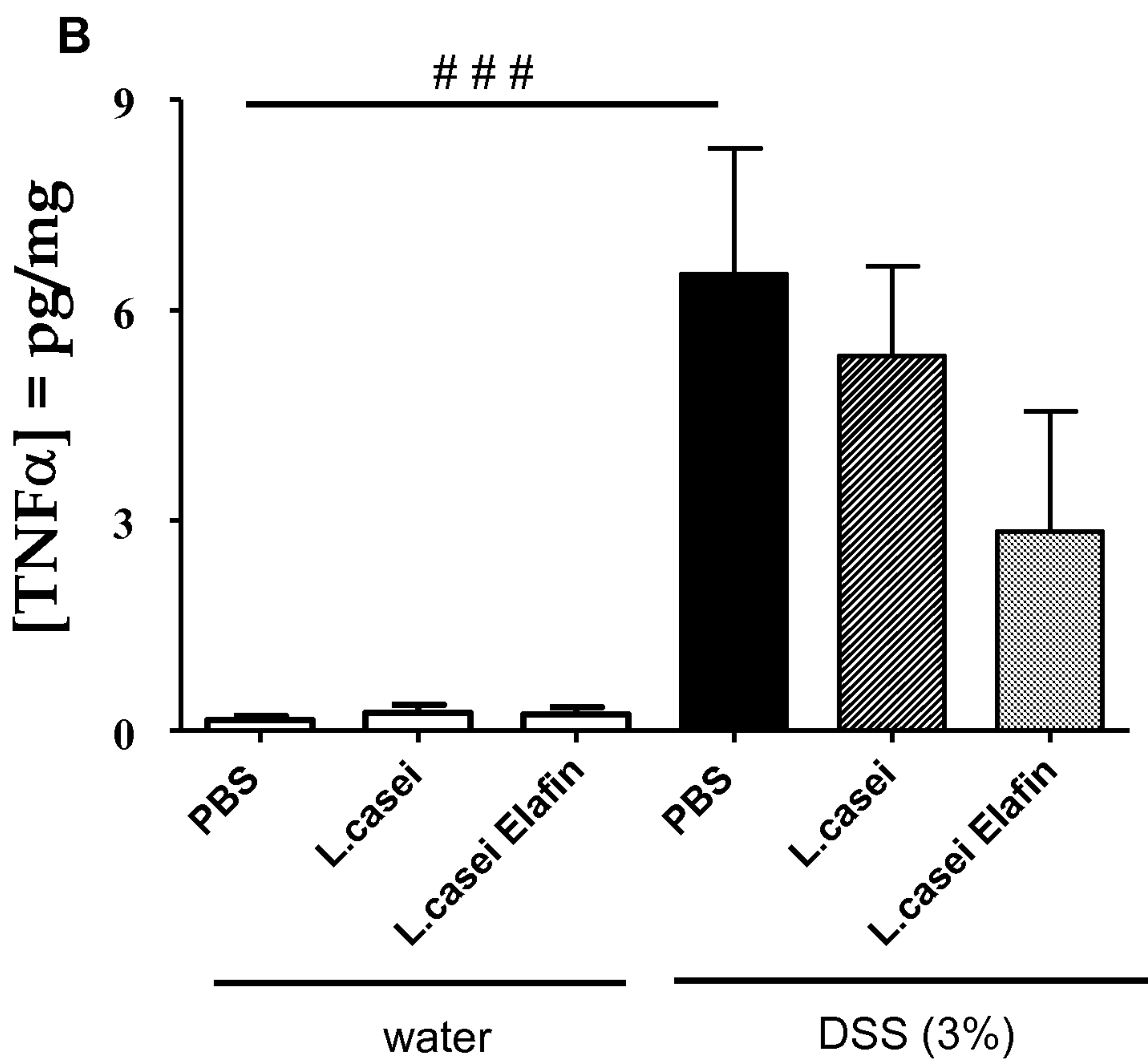
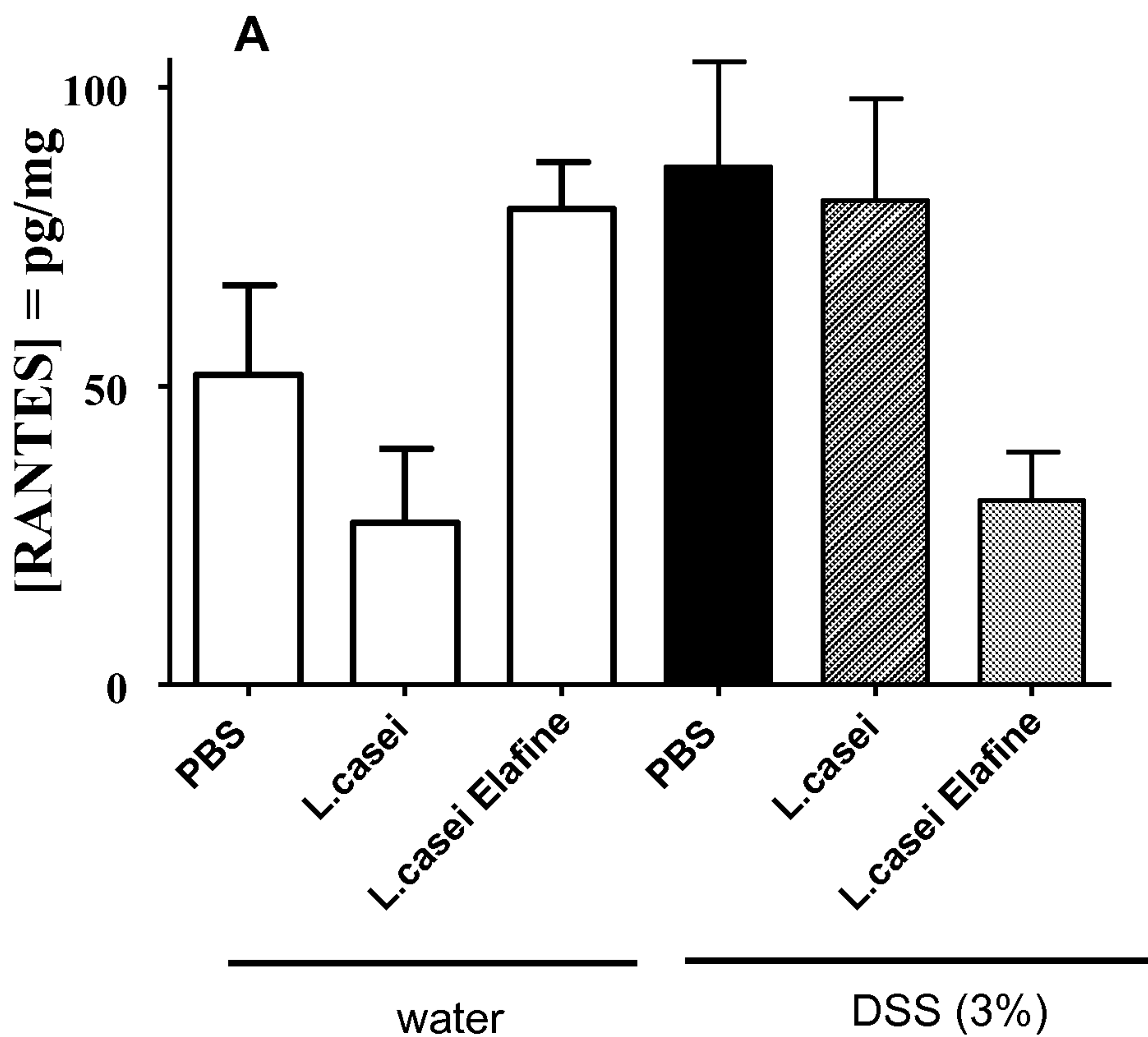
Figure 3 C

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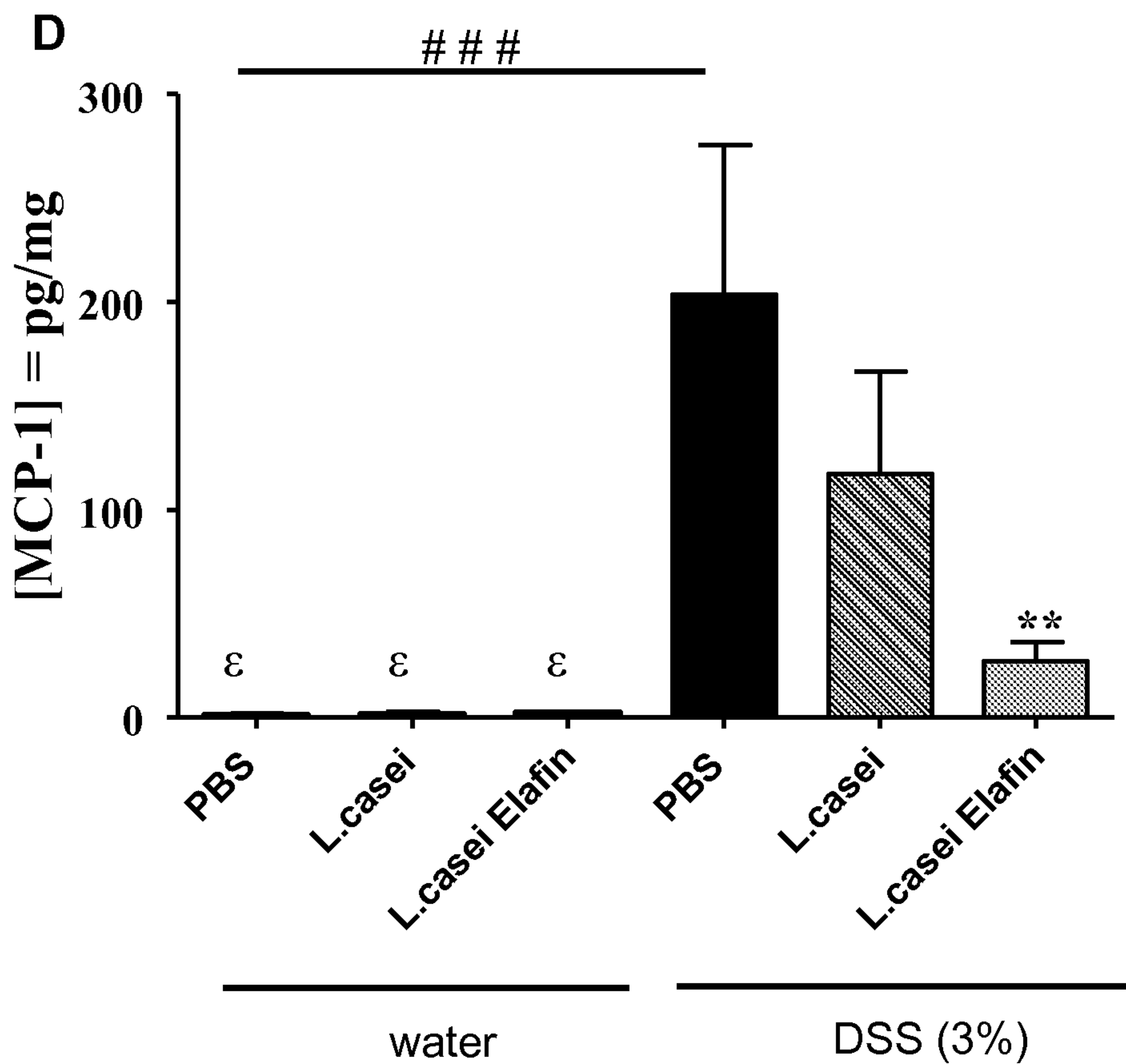
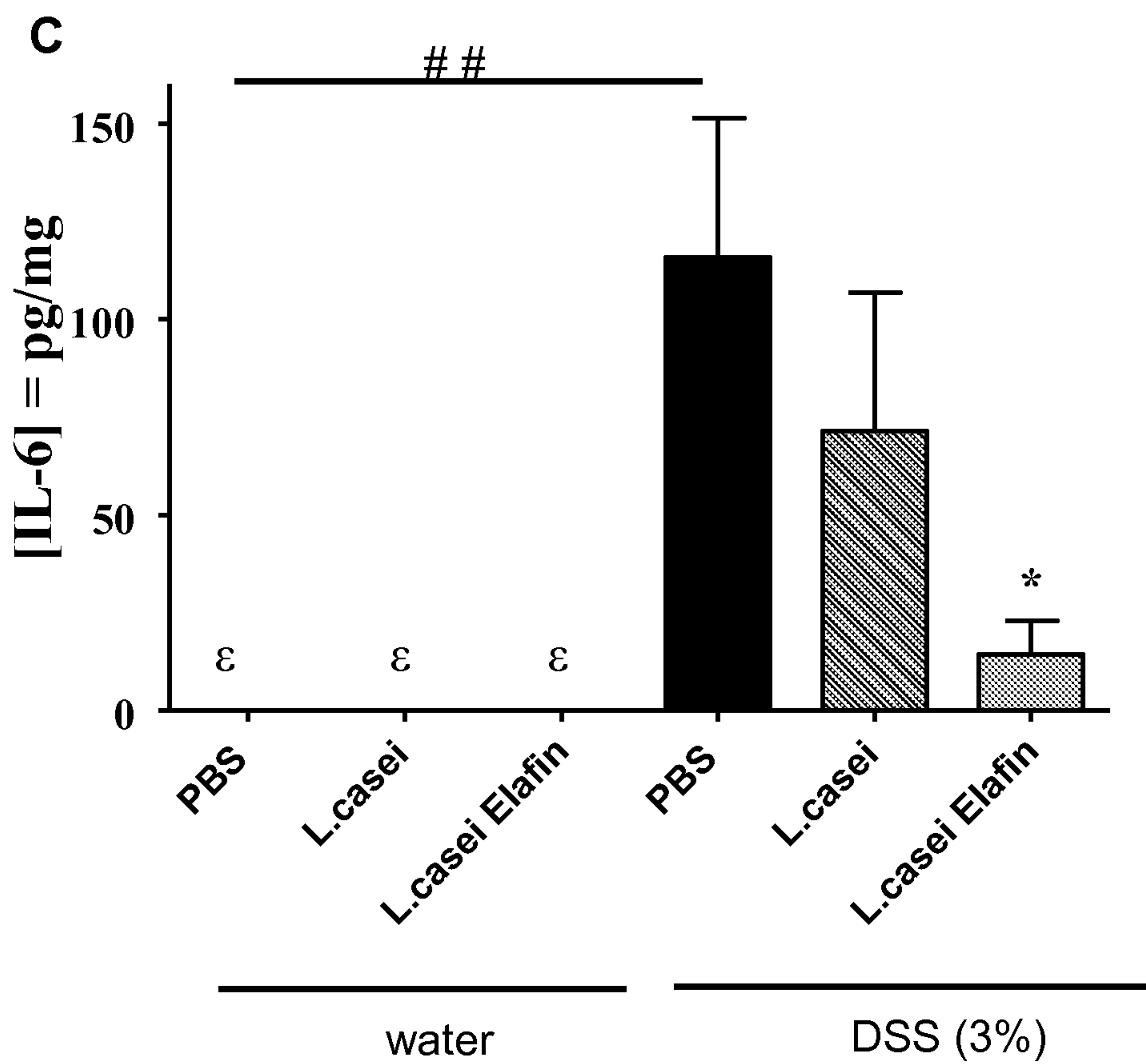
Figures 4 A and B

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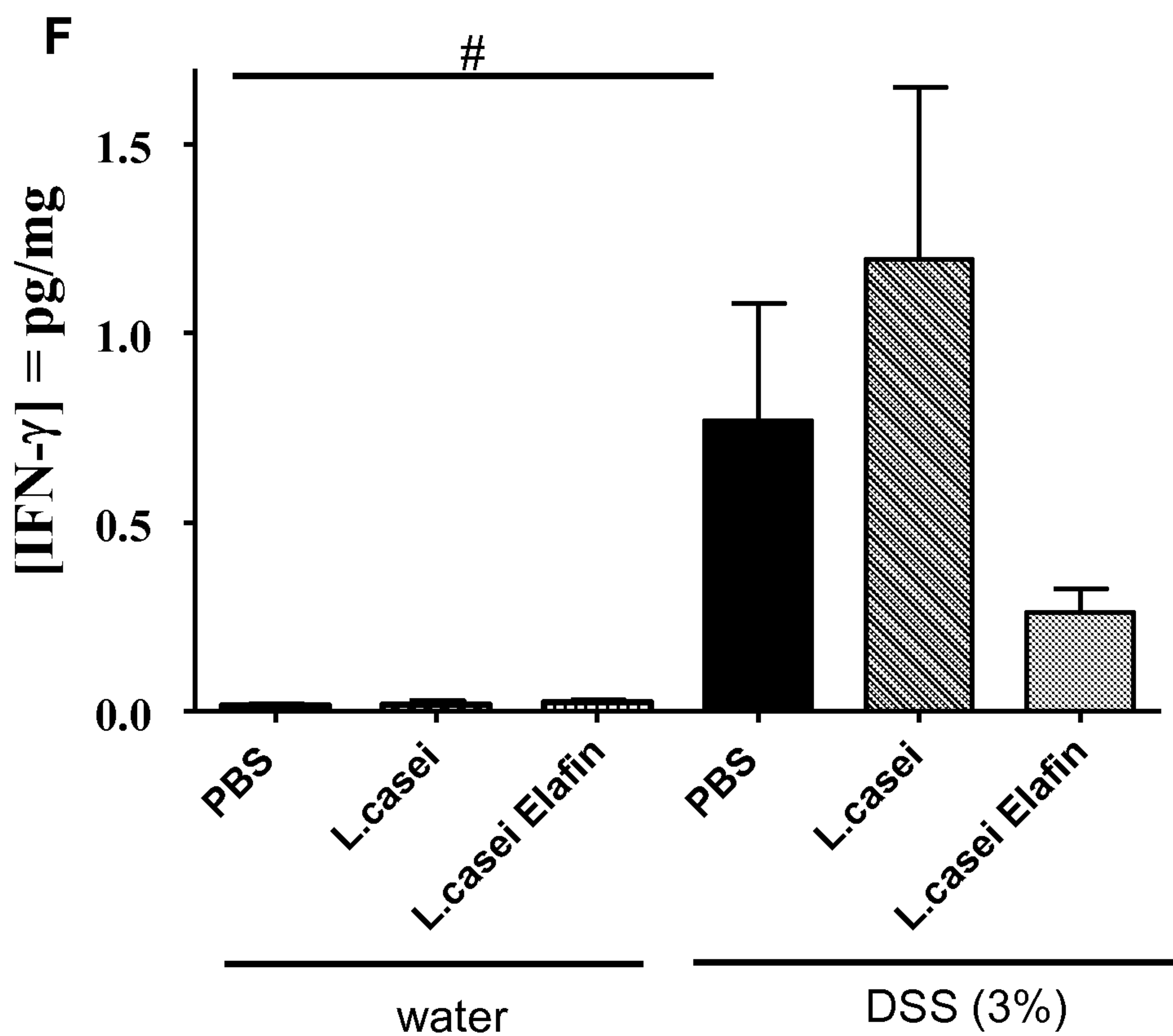
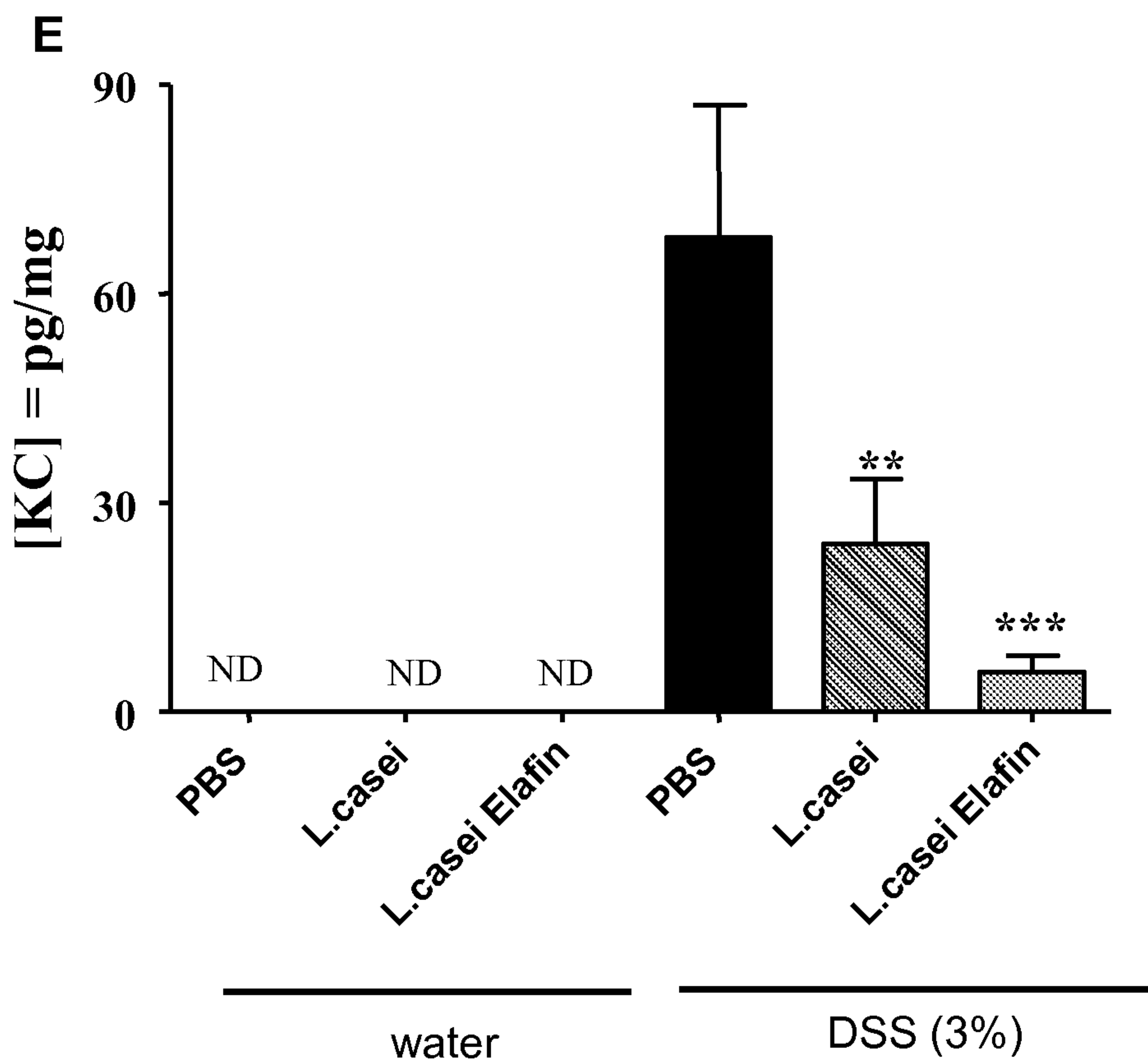
Figures 5 A and B

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Figures 5 C and D

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Figures 5 E and F

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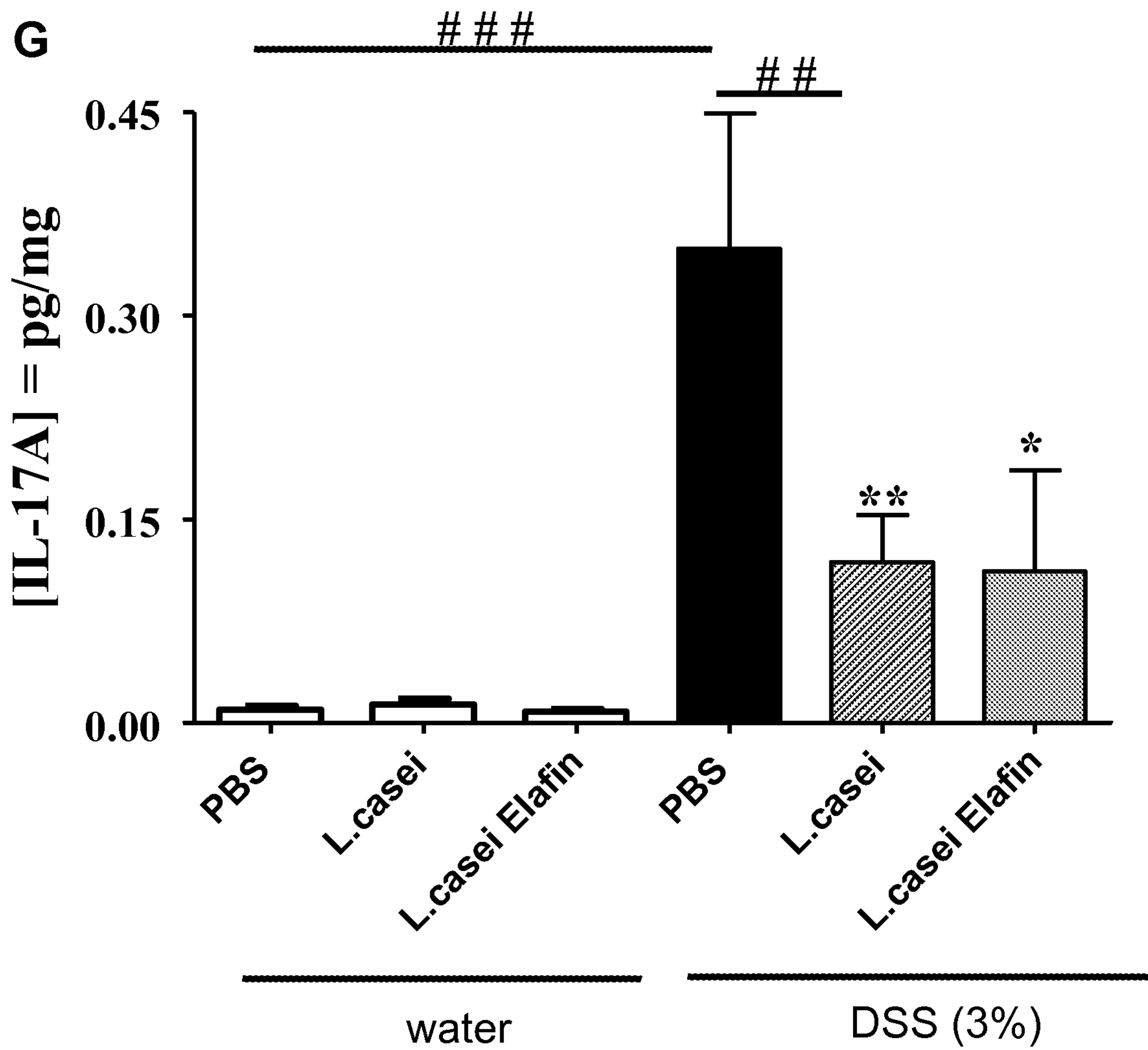
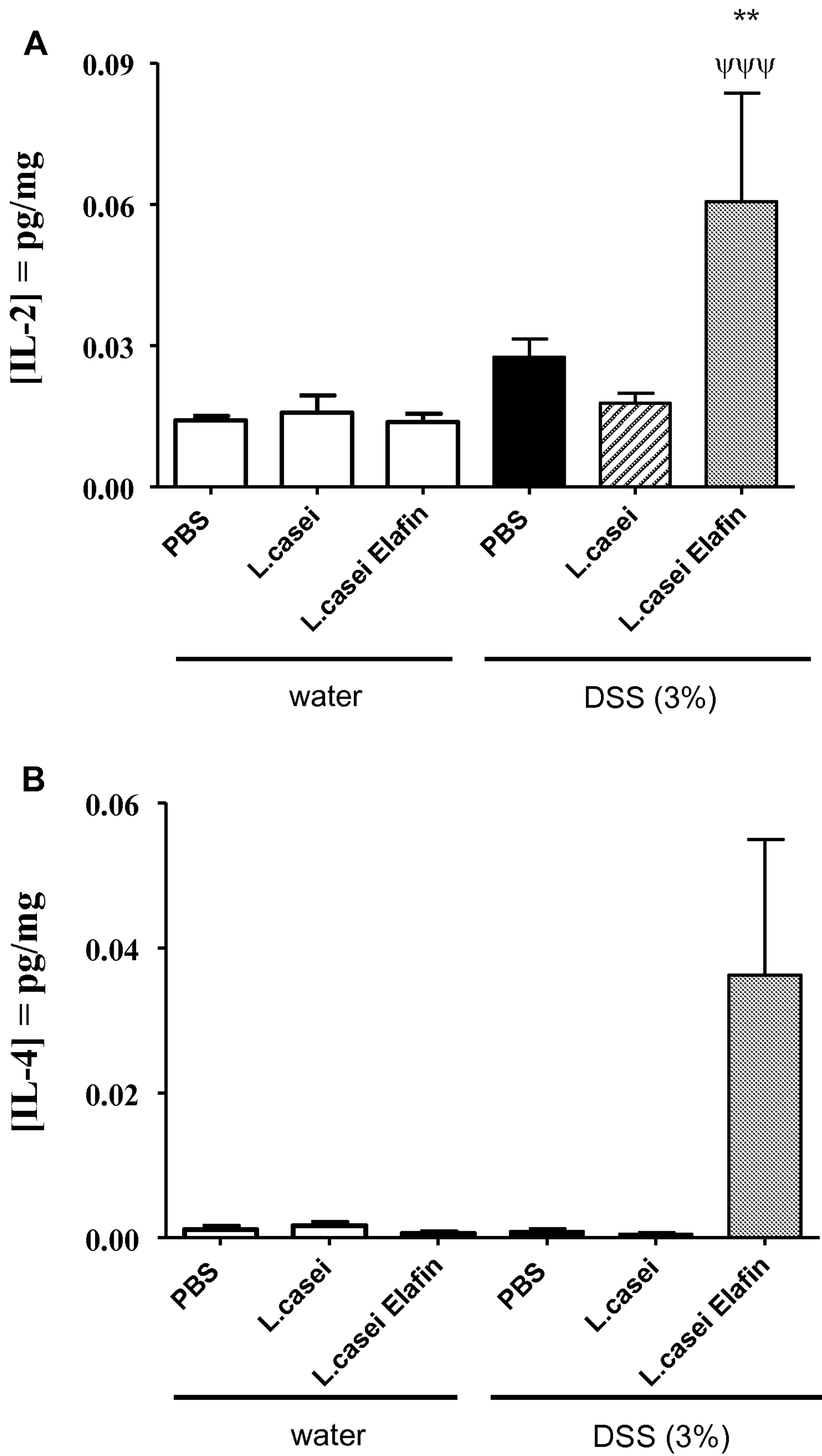


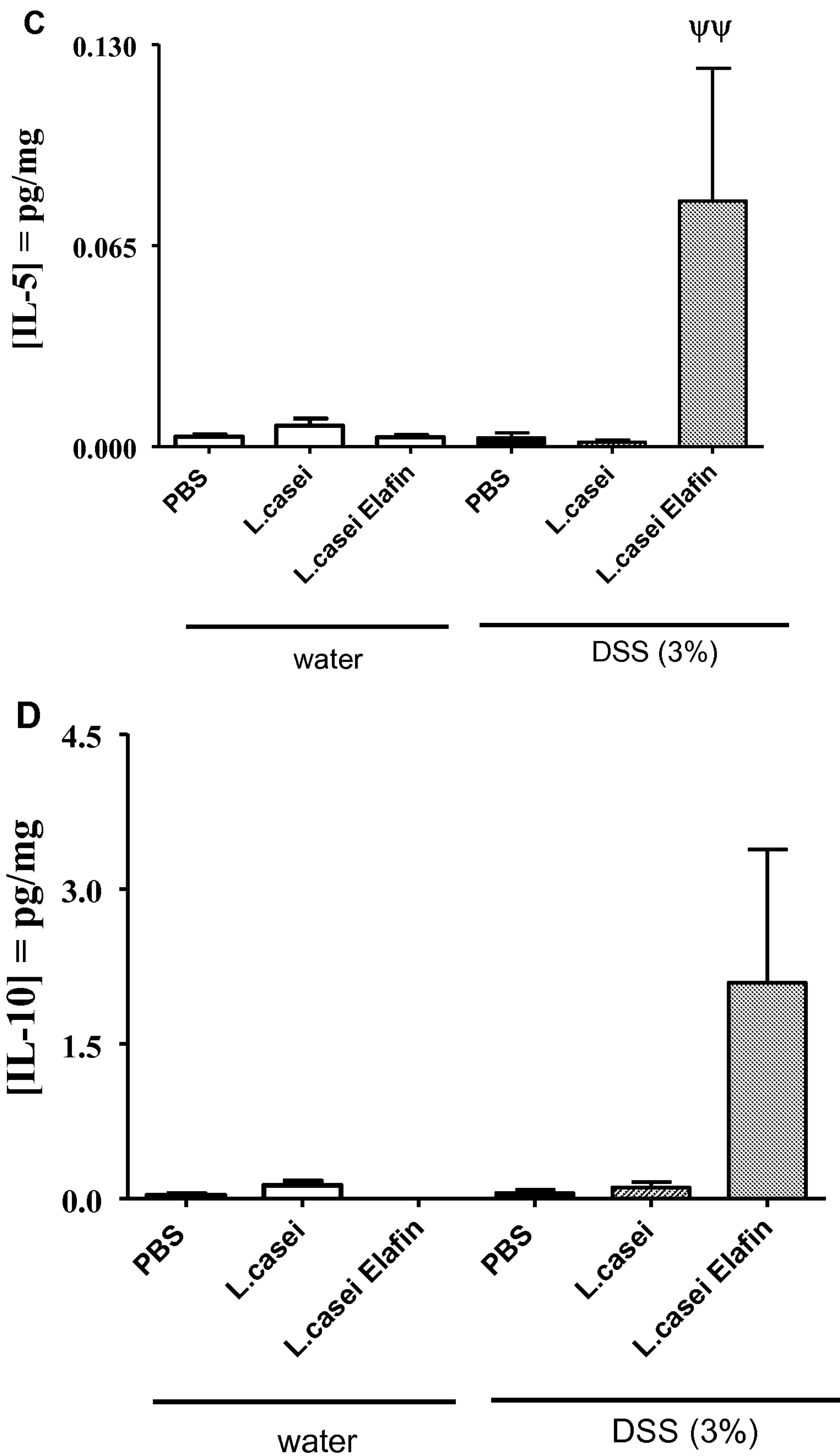
Figure 5 G

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Figures 6 A and B

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Figures 6 C and D

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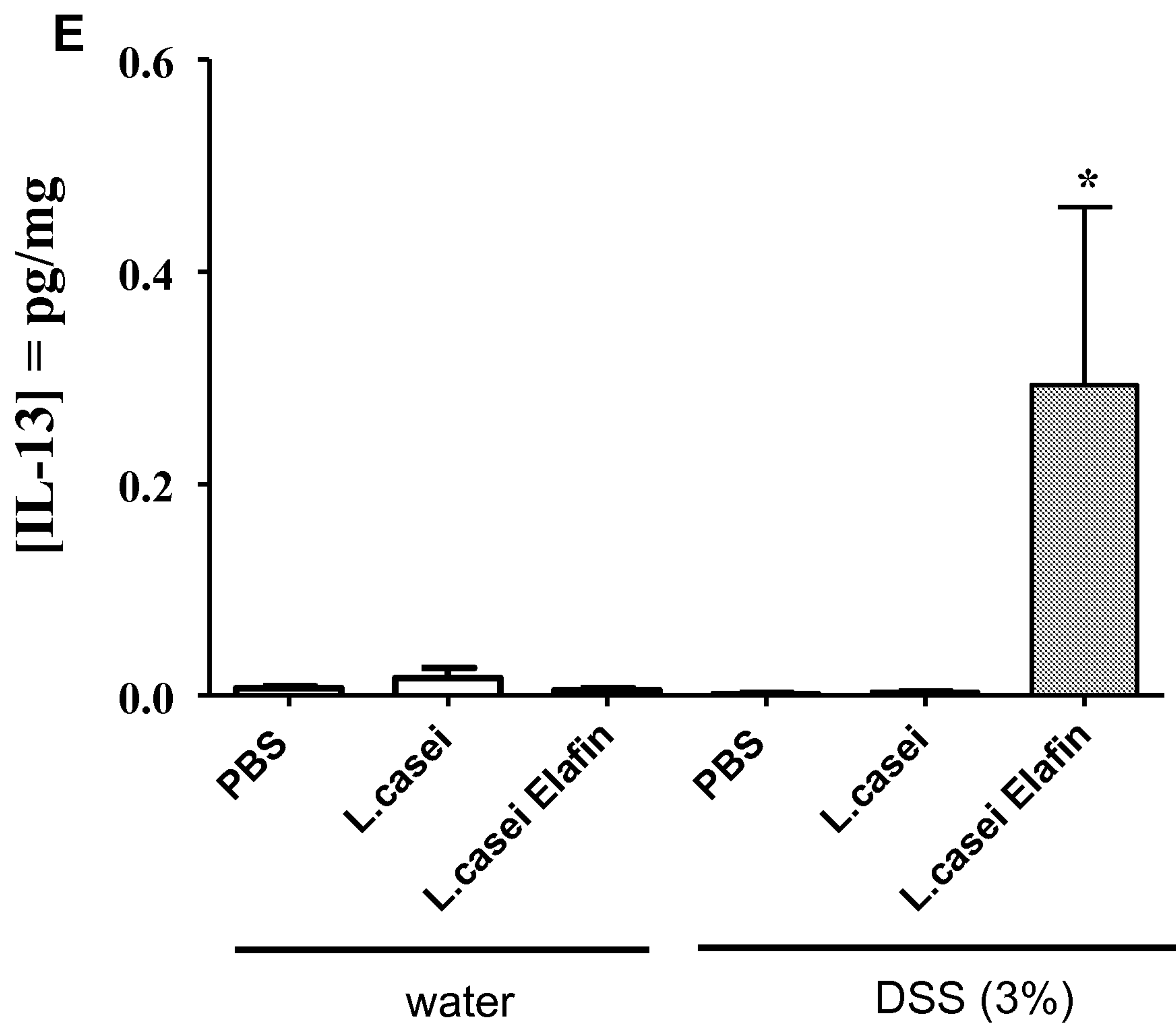
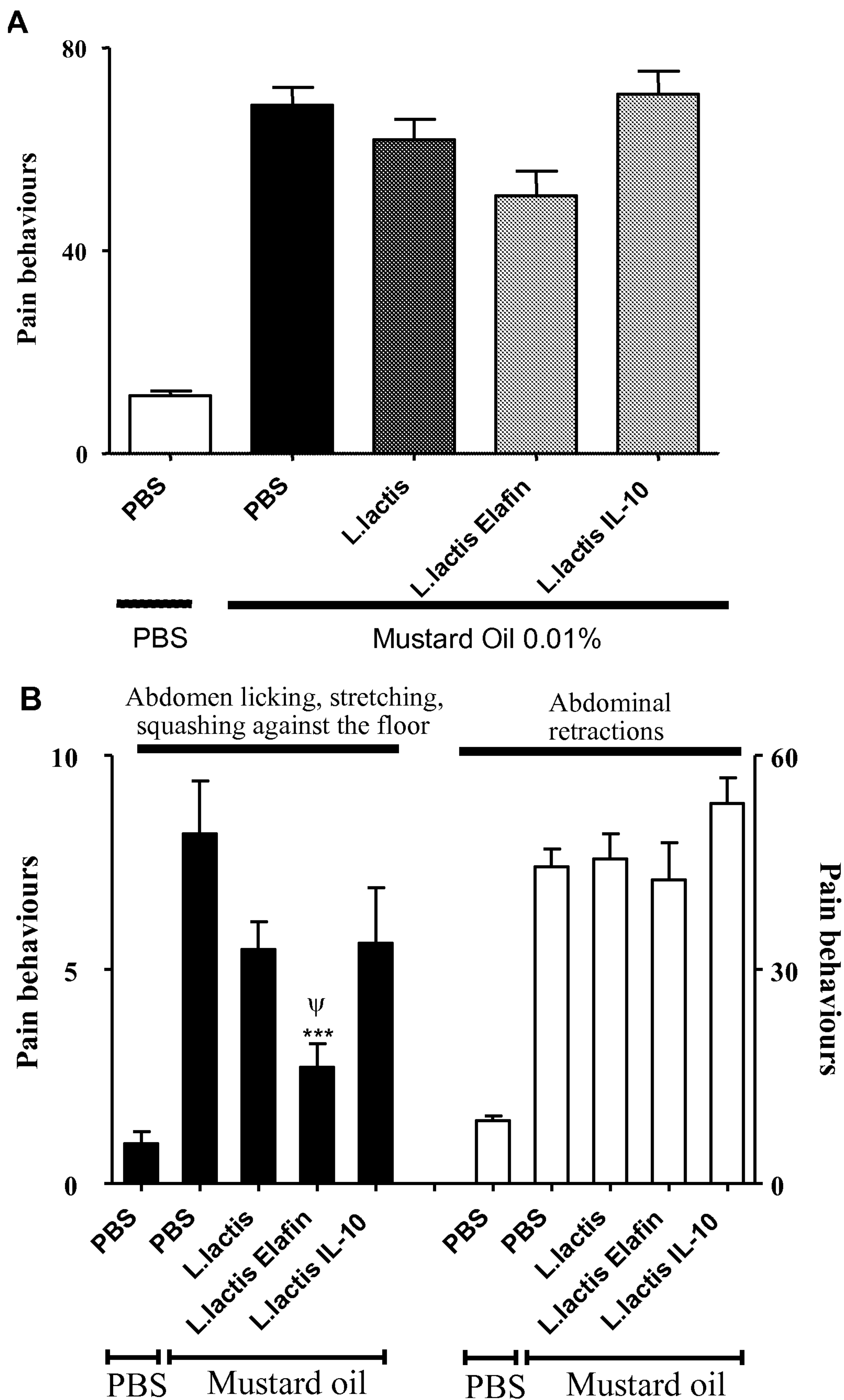


Figure 6 E

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Figures 7 A and B

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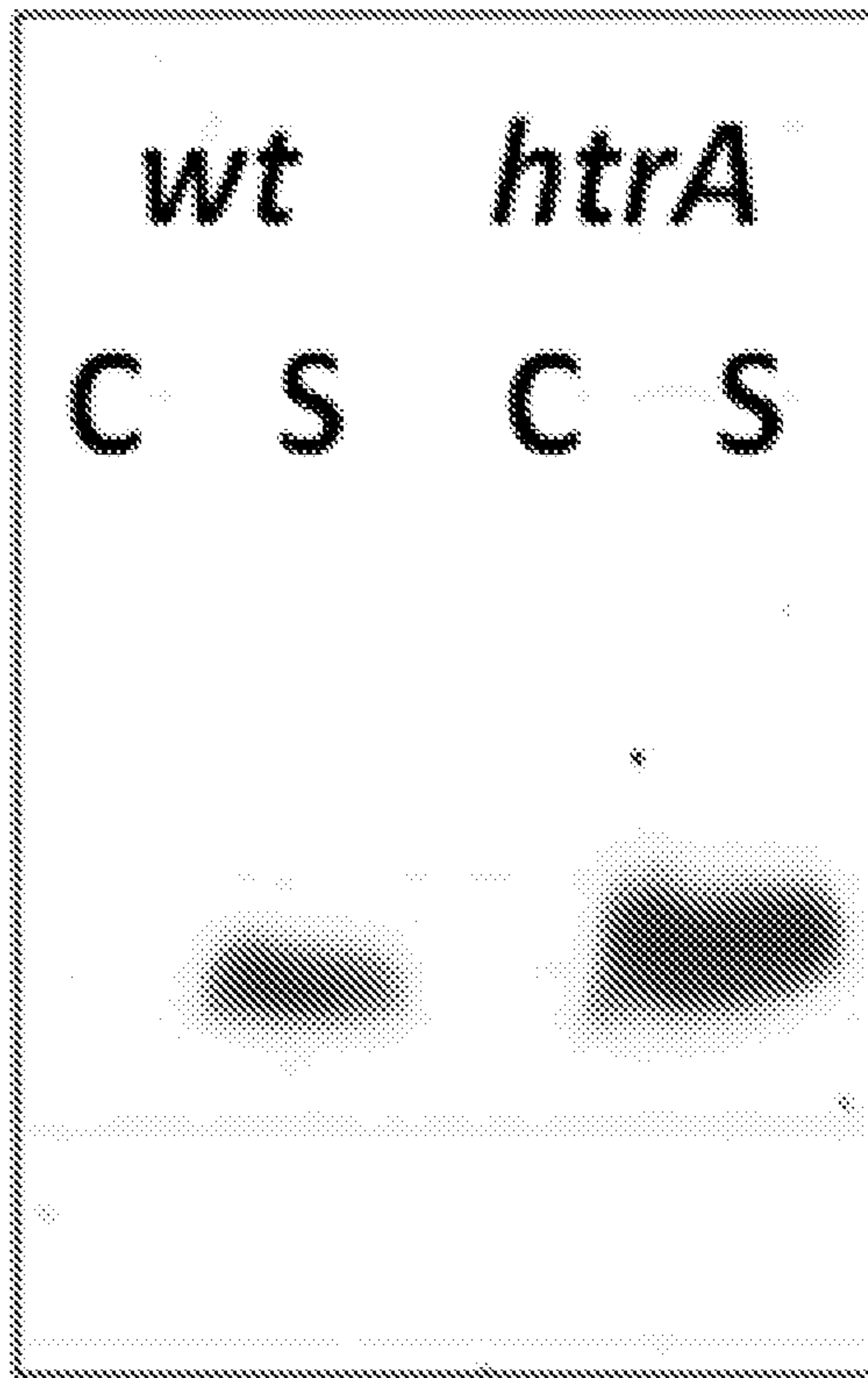


Figure 8

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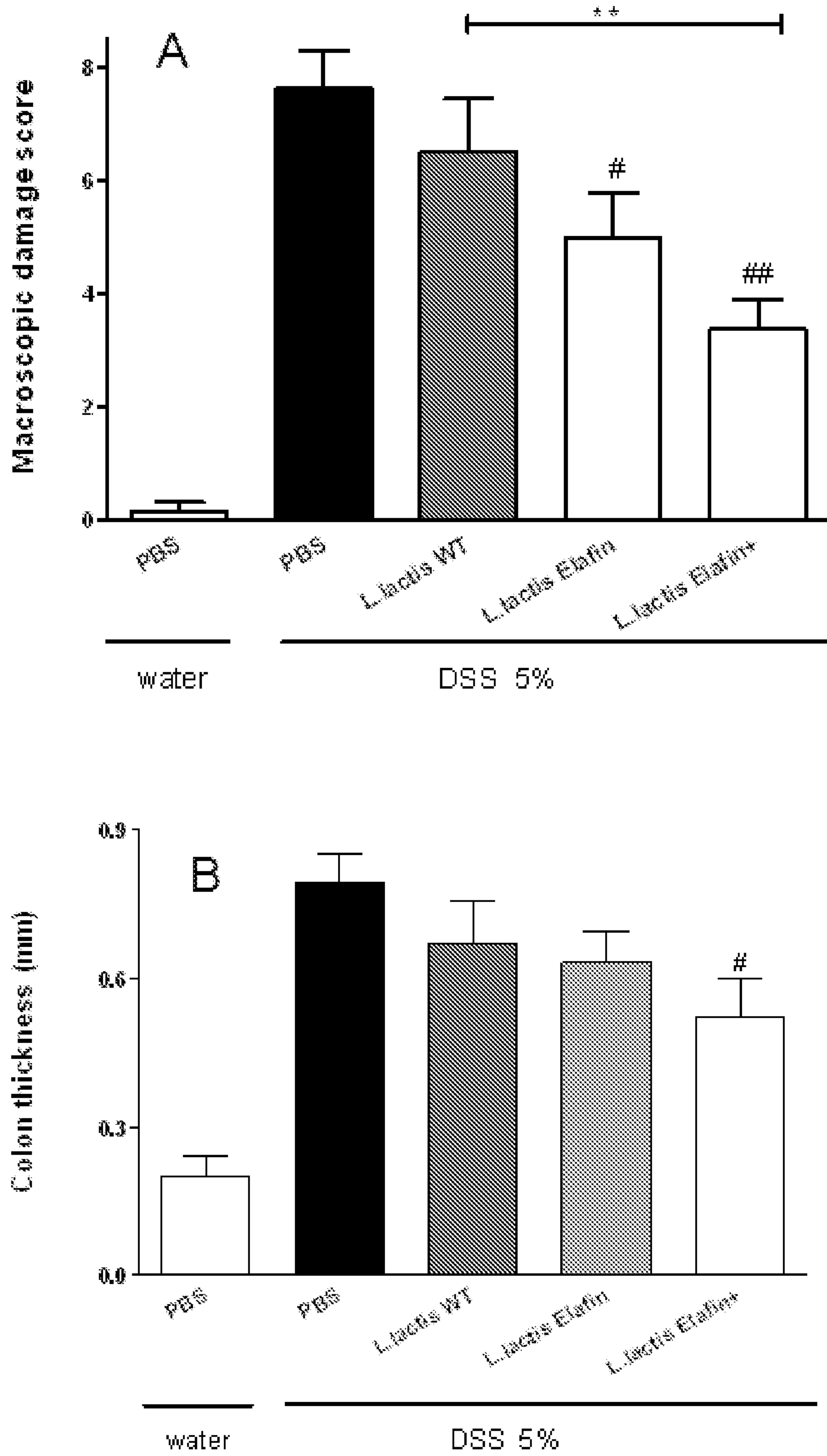


Figure 9 A and B

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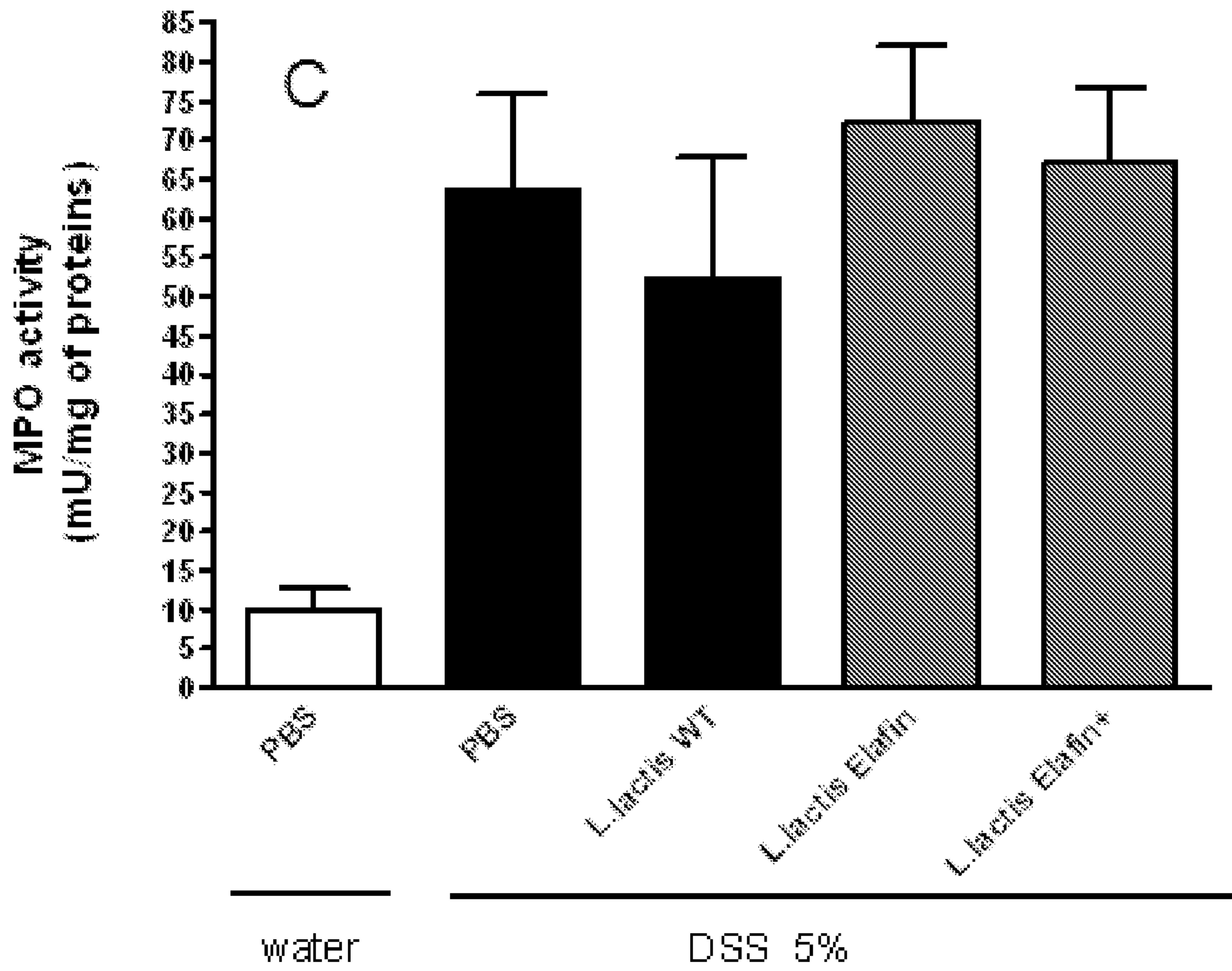
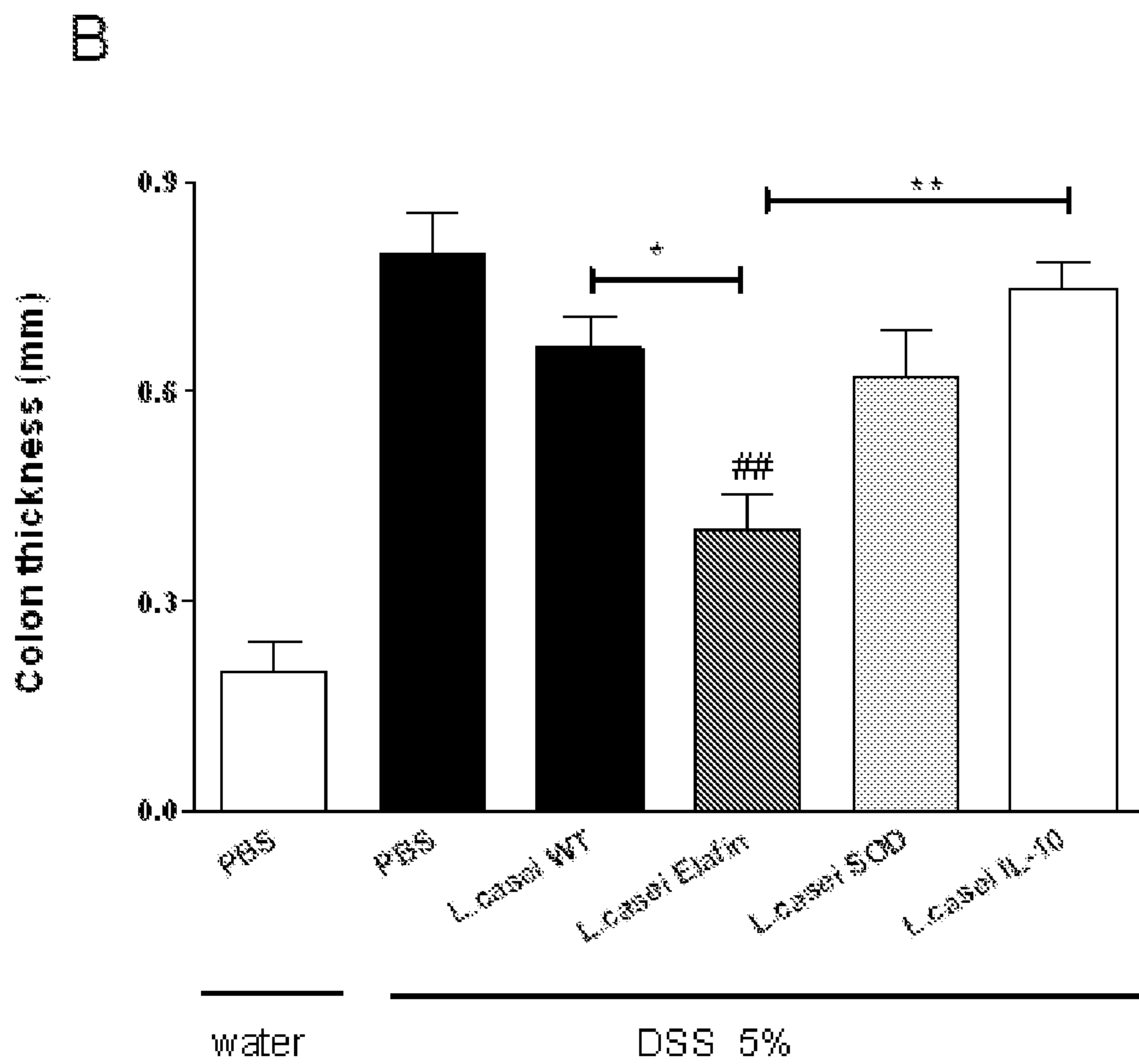
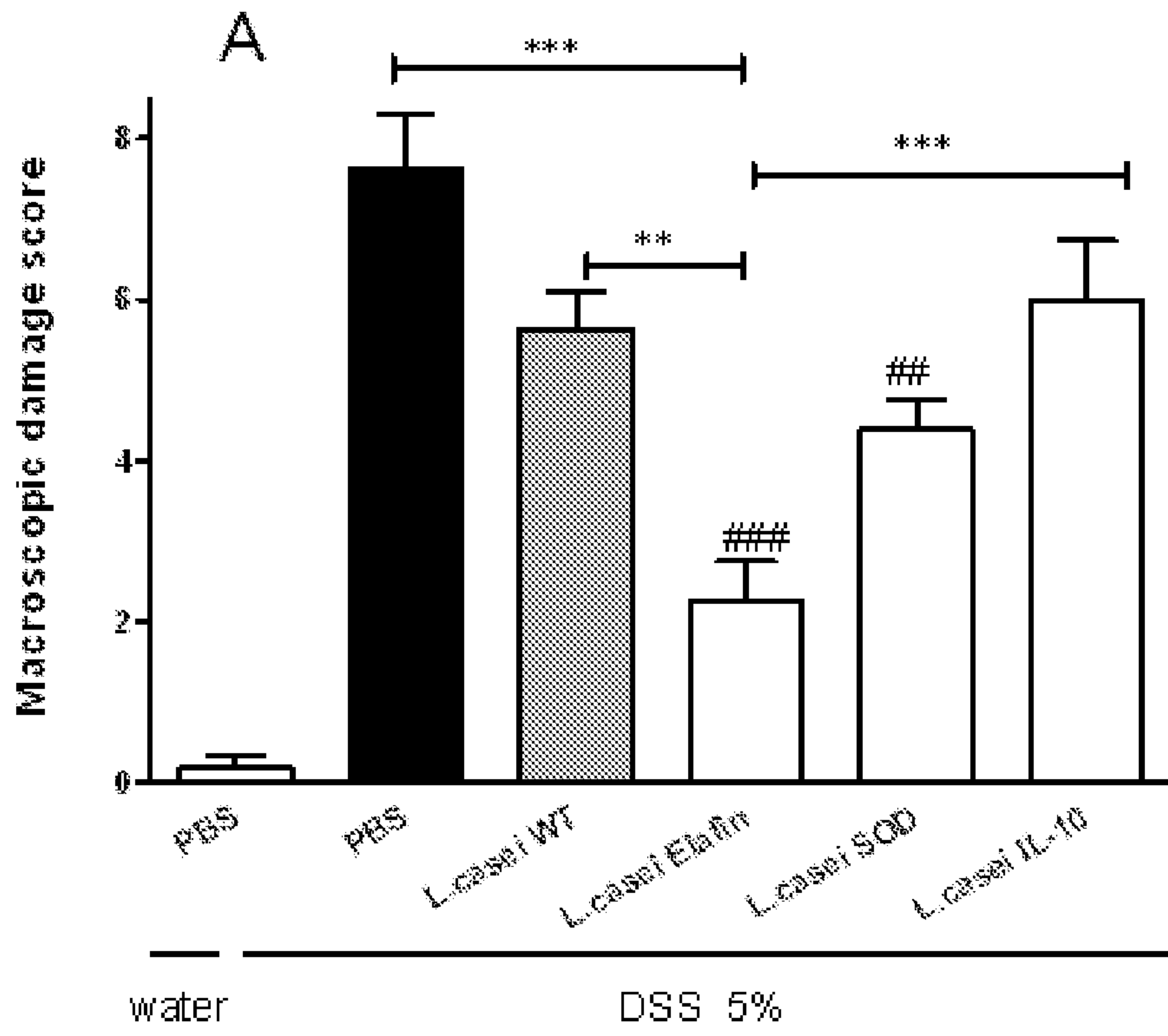


Figure 9 C

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Figures 10 A and B

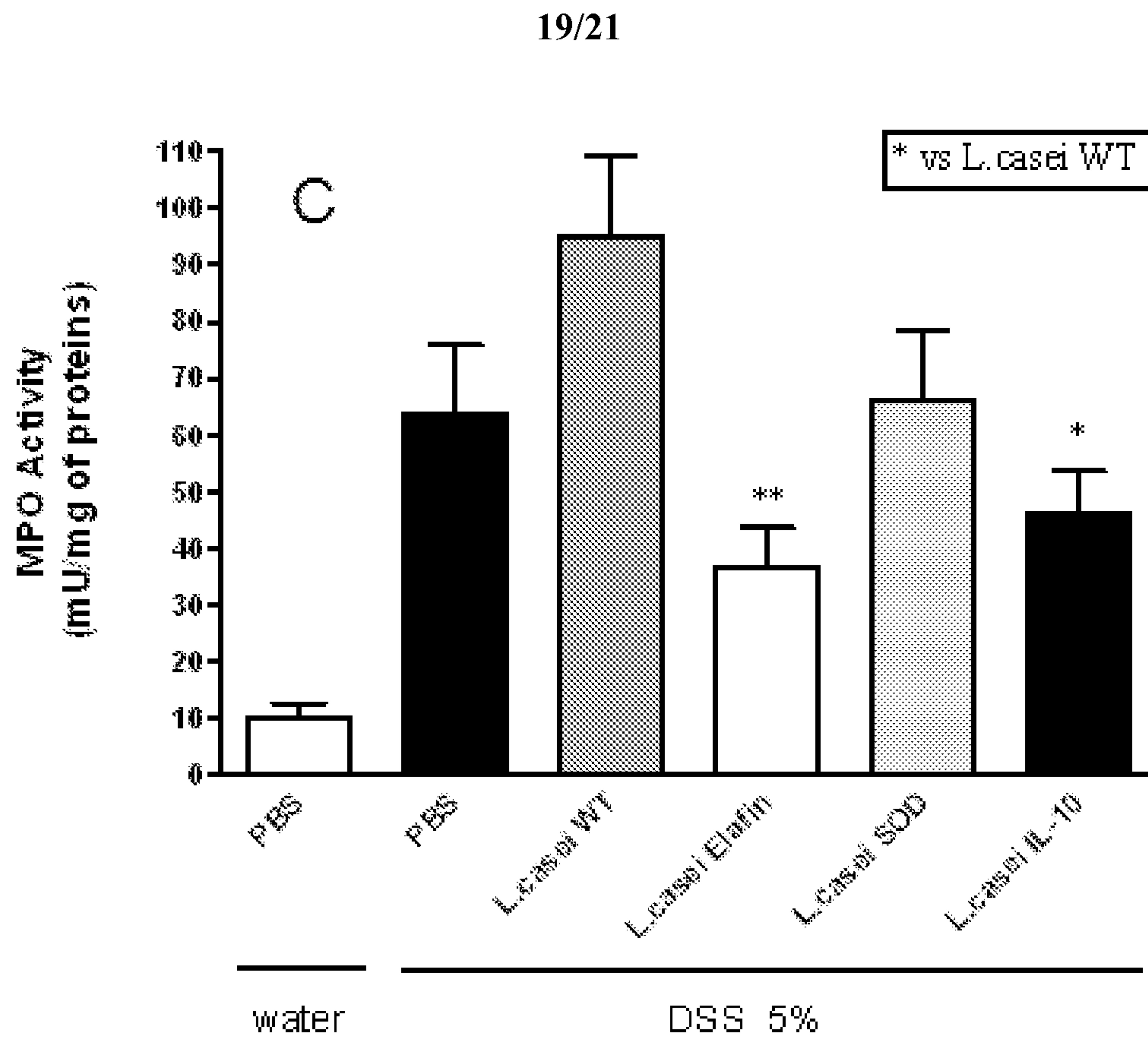


Figure 10 C

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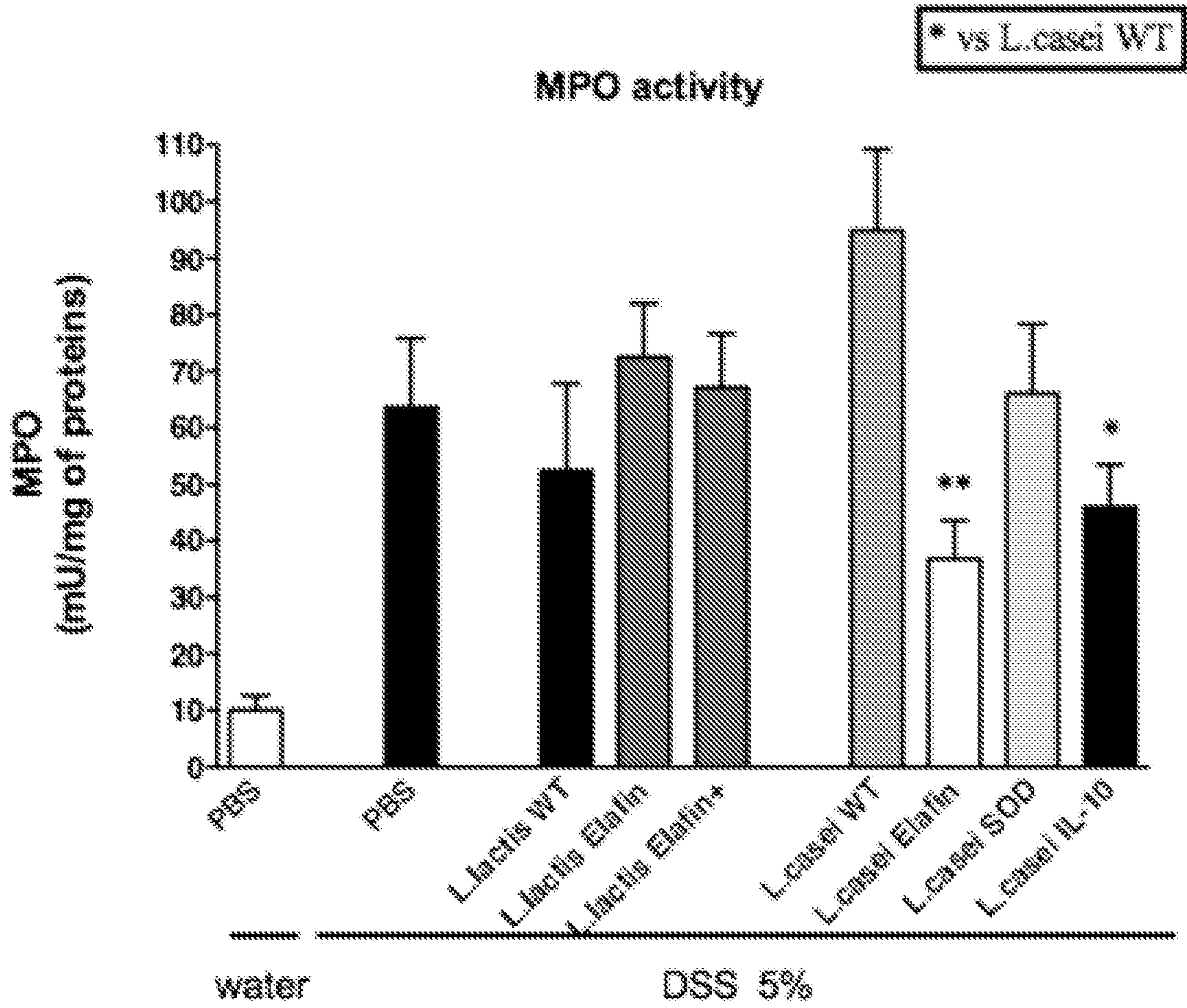


Figure 11

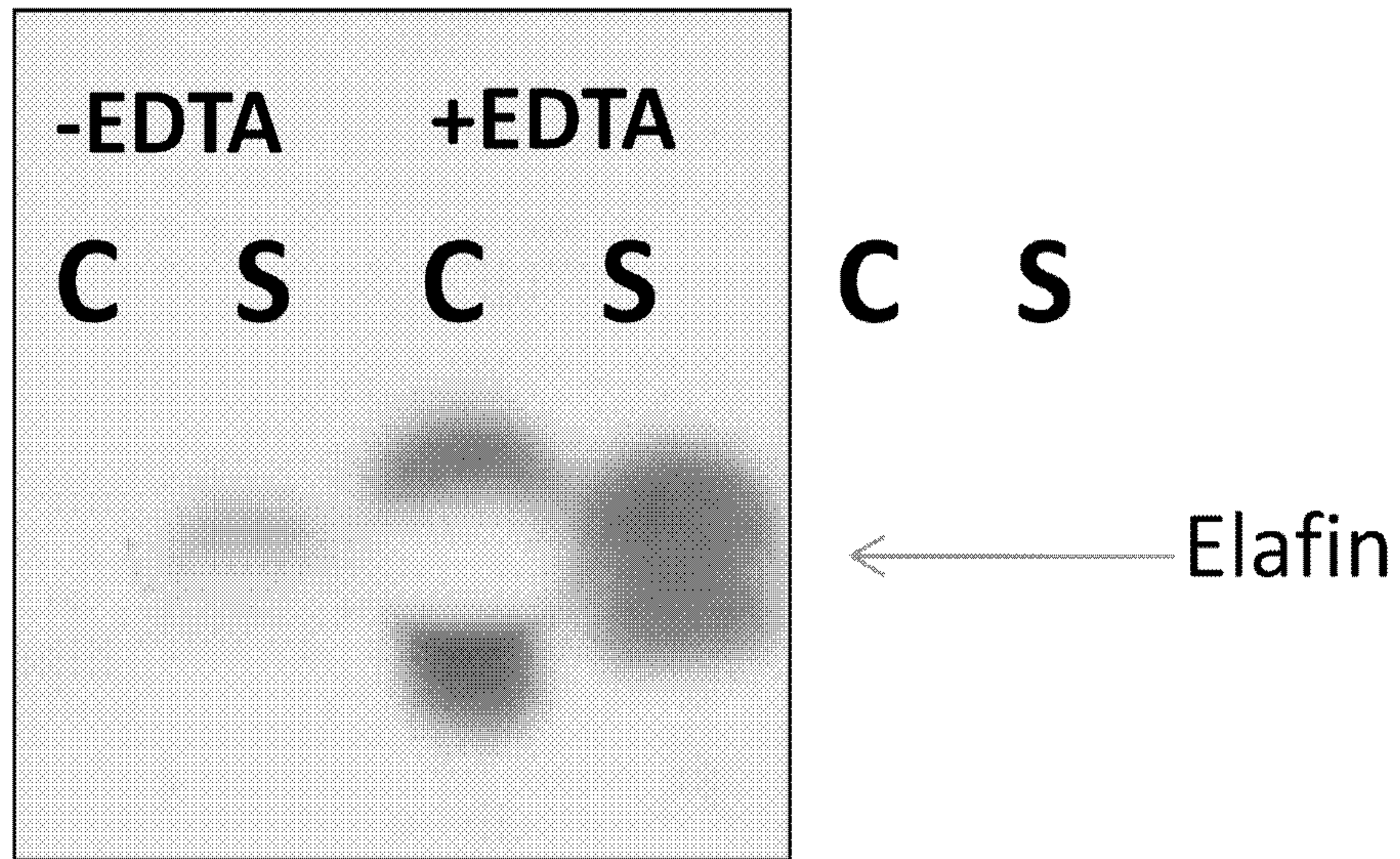


Figure 12