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# Immunomodulatory Effect of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus Strains* of Paraprobiotics in Lipopolysaccharide-Stimulated Inflammatory Responses in RAW-264.7 Macrophages

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## Abstract

The discovery of the potential of paraprobiotics to exert different immunological benefits suggests that further studies should be carried out to determine their potential and mechanisms of action in modulating the immune system. The objective of this study was to investigate the immune response of several microbial-associated molecular patterns (MAMPS) used at different doses in macrophage cell lines RAW-264.7 stimulated with lipopolysaccharide (LPS). Two experiments were conducted. The first was performed to determine a dose response curve for each paraprobiotic (*Bifidobacterium lactis*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, and *Streptococcus thermophilus)*. Further experiments were carried using only two doses (0.01 g/ml and 0.1 g/ml). RAW-264.7 cells were cultivated in Dubelcco's Modified Eagle's medium supplemented with fetal bovine serum and penicillin/streptomycin. Cells were incubated with LPS (1 µg/ml) and six concentrations of MAMPs were added. RAW-264.7 viability, myeloperoxidase activity, nitrite/nitrate concentration, reactive oxygen species production, oxidative damage, and inflammatory parameters were measured. In the LPS group, there was a significant reduction in cell viability. Myeloperoxidase and nitrite/nitrate concentrations demonstrated a better effect at 0.01 and 0.1 g/ml doses. There was a significant reduction in interleukin-6 (IL-6) levels at 0.1 g/ml dose in all paraprobiotics. IL-10 levels decreased in the LPS group and increased at 0.1 g/ml dose in all paraprobiotics. The dichlorofluorescin diacetate results were reinforced by the observed in oxidative damage. Paraprobiotics are likely to contribute to the improvement of intestinal homeostasis, immunomodulation, and host metabolism.

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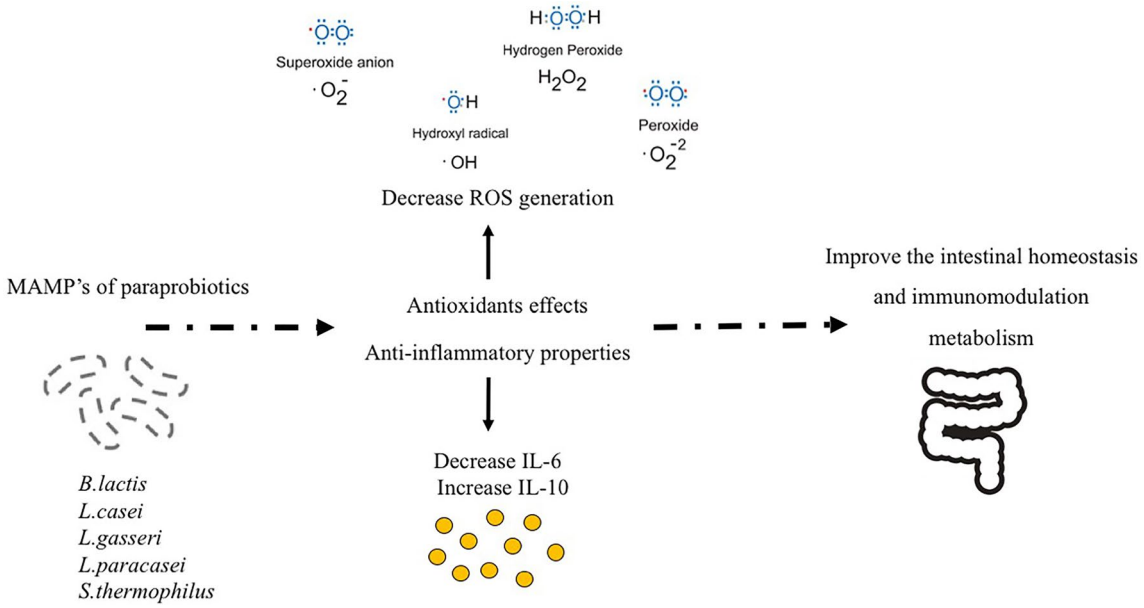
**Graphical Abstract**




## Introduction

The health-promoting effects of probiotics have already been verified through a series of studies [1]. However, although less studied, a considerable amount of data has revealed the beneficial effects of paraprobiotics, indicating that non-viable microbial cells, microbial fractions, or cell lysates can also immunomodulate human and animal health [2].

Paraprobiotics are defined as "inactivated (non-viable) microbial cells or cellular fractions that, when consumed, confer benefits to the consumer's health" [2]. A number of benefits associated with the consumption of paraprobiotics have already been verified, such as: immune system modulation [3], treatment of liver disease [4], reduction of diarrhea symptoms [5, 6], atopic dermatitis [7], and colitis [8], inhibition of pathogens [9, 10], prevention of dental caries [11], modulation of the intestinal microbiota; maintenance of intestinal integrity [4], and cholesterol reduction [12] in addition to reducing flatulence and food allergy risks [13, 14]. It is important to note that paraprobiotics are different from postbiotics since postbiotics refer to soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis, such as enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins, and organic acids [4].

Communication with the host can be mediated by bacterial cells and is based on the activation of the innate immune response through interaction with Toll-like receptors (TLRs) [2]. Toll-like receptors form a family of receptors that recognize molecular patterns often associated with infectious agents [15]. Among the different receptors is TLR4, which recognizes bacterial components, such as lipopolysaccharide (LPS), of gram-negative bacteria [16, 17].

In general, after the interaction among paraprobiotics, also known as microbe-associated molecular patterns (MAMPs), and pattern recognition receptors (PPRs), the innate immune response is activated to coordinate a response involving humoral and cellular components. In this context, after MAMPs-PPRs interaction, resident cells play a key role, releasing a wide variety of signaling molecules, such as prostaglandins, leukotrienes, cytokines, and chemokines that trigger the inflammatory response, representing one of the most important functions of innate immunity [18, 19].

Inflammation and oxidative stress are important mechanisms of this response, as they consist of a variety of physiological and pathological events, acting as a permissive mechanism for the action of cells and components of the immune system [20]. The intensity of the immune response and the harmful potential of the stimulating agent are directly proportional to the severity of the effects observed in the affected individuals [21].

The discovery of the potential of paraprobiotics to exert different immunological benefits suggests that further studies should be carried out to determine the potential and mechanisms of action of these compounds in modulating the immune system. Thus, the objective of this study was to

investigate the response of several MAMPs (*Bifidobacterium lactis*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus paracasei* and *Streptococcus thermophilus)* at different doses in a lineage of RAW-264.7 macrophages stimulated with LPS to determine its anti-inflammatory effect that could be used to mitigate inflammation in different diseases.

## Material and Methods

### Reagents

Heat-inactivated MAMPs of *Bifidobacterium animalis* subsp. *lactis* CCT 7858, *Lactobacillus casei* CCT 7859, *Lactobacillus gasseri* CCT 7860, *Lactobacillus paracasei* subsp. *paracasei* CCT 7861, and *Streptococcus thermophilus* ATCC 19258 were provided by Gabbia Biotechnology. For the production of MAMPs, probiotic microorganisms were grown in specific culture media. After confirming their growth, 5 mL tubes containing cell suspension of probiotic microorganisms were inactivated through a thermal process (time and temperature defined in the protocol of company Gabbia Biotecnologia). When inactivation was confirmed, MAMPs were used in the "in vitro" tests.

Lipopolysaccharide (LPS from *Escherichia coli* 026:B6) was obtained from Sigma Chemical Co (St. Louis, MO, EUA) and was used at 1 µg/ml [22]. The LPS was reconstituted in endotoxin-free water. A murine strain of RAW-264.7 macrophages was obtained from Sigma Chemical Co (St. Louis, MO, EUA, RAW-264.7 Cell Line murine: 91062702). Dulbecco's Modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), streptomycin (0.1 mg/ml) and penicillin (100 U/ml) was obtained from Sigma Chemical Co (St. Louis, MO, EUA). Fetal Bovine Serum (FBS) was obtained from Gibco BRL—Life Technologies (Rockville, MO, EUA).

### Cell Culture

RAW-264.7 cells were cultivated in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin under 5% $CO_2$ humidified conditions at 37 °C [23]. The cells were seeded at $1 \times 10^4$ cells/well in a 96-well plate and maintained for 24 h. After this period, the cells were incubated with LPS (1 µg/ml) for an additional 24 h, after which the cells were treated with different MAMPs for 24 h.

### Dose Response Experiments

First, a dose response curve for each paraprobiotic was determined. Paraprobiotics at six concentrations (0.0001 g/ml; 0.001 g/ml; 0.01 g/ml; 0.1 g/ml; 1 g/ml; 2 g/ml) were added after LPS incubation as follows:

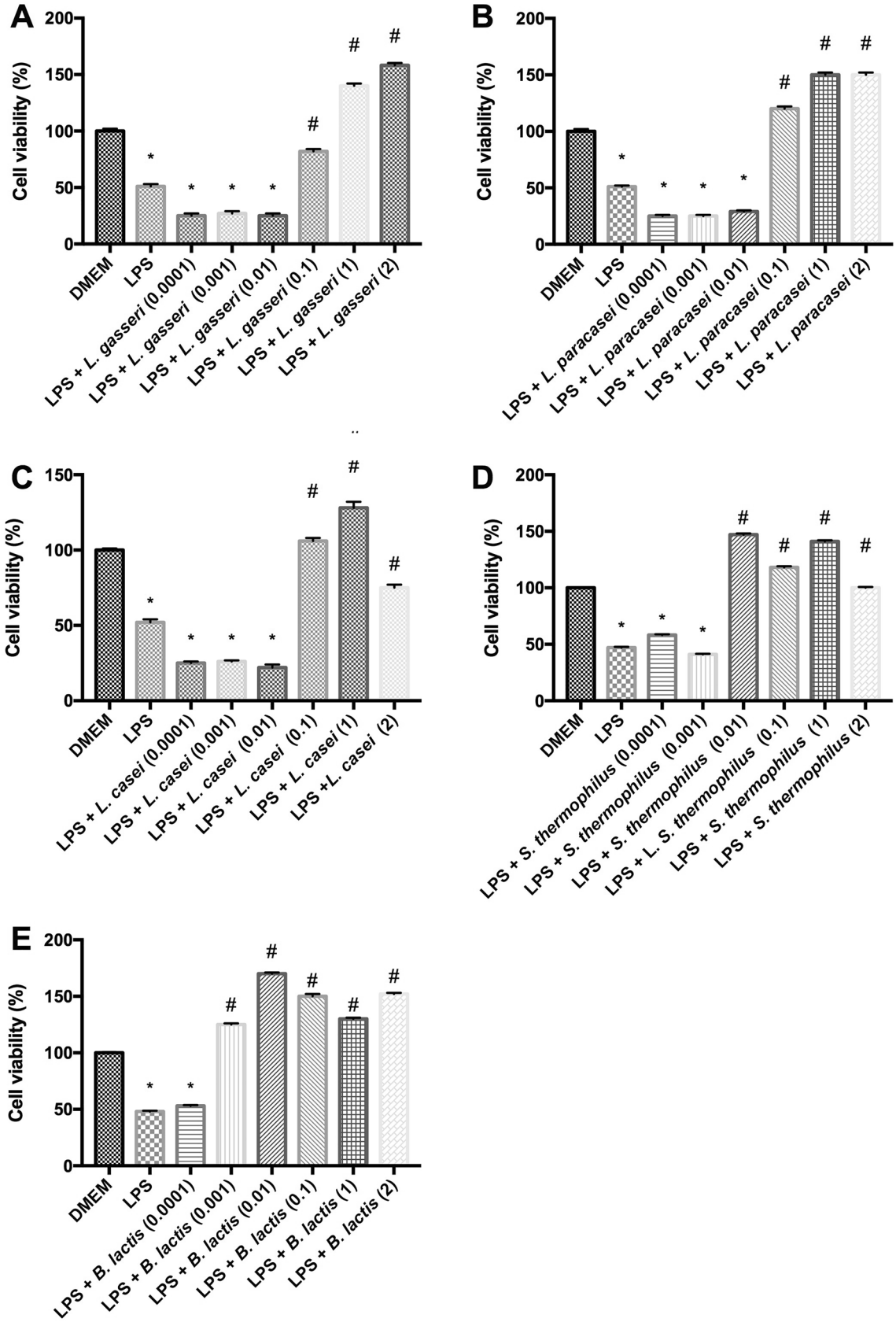
(1) RAW-264.7 + DMEM (control group).
(2) RAW-264.7 + LPS.
(3) RAW-264.7 + LPS + MAMPs of *Bifidobacterium lactis* (6 different doses).
(4) RAW-264.7 + LPS + MAMPs of *Lactobacillus casei* (6 different doses).
(5) RAW-264.7 + LPS + MAMPs of *Lactobacillus gasseri* (6 different doses).
(6) RAW-264.7 + LPS + MAMPs of *Lactobacillus paracasei* (6 different doses).
(7) RAW-264.7 + LPS + MAMPs of *Streptococcus thermophilus* (6 different doses).

All experiments were performed in triplicate and four wells were used for each condition. RAW-264.7 viability, nitrite/nitrate concentration, and myeloperoxidase (MPO) activity were evaluated as described below ("Effects of paraprobiotics in ROS generation, oxidative damage, and inflammatory parameters", "Oxidative Damage", and "Nitrosative damage", respectively). After determining the best dose–response for each paraprobiotic, further experiments were carried out as described below.

### Effects of Paraprobiotics in ROS Generation, Oxidative Damage, and Inflammatory Parameters

RAW-264.7 cells were plated in a 96-well plate and stimulated with LPS (1 µg/ml), as described in experimental design 1. Then, MAMPs were added at 0.01 g/ml and 0.1 g/ml for 24 h (LPS have been maintained in culture). Since, maltodextrin was used during the production of MAMPs, there is a possibility that its traces could be present in used MAMPs. Thus, it was included as an additional control group, just to ensure that it did not have any substantial effect in our model.

(1) RAW-264.7 + DMEM (control group).
(2) RAW-264.7 + LPS.
(3) RAW-264.7 + Maltodextrin (additional control—see text).
(4) RAW-264.7 + LPS + MAMPs of *Bifidobacterium lactis* (2 different doses).
(5) RAW-264.7 + LPS + MAMPs of *Lactobacillus casei* (2 different doses).
(6) RAW-264.7 + LPS + MAMPs of *Lactobacillus gasseri* (2 different doses).
(7) RAW-264.7 + LPS + MAMPs of *Lactobacillus paracasei* (2 different doses).
(8) RAW-264.7 + LPS + MAMPs of *Streptococcus thermophilus* (2 different doses).


A
Cell viability (%)
200
150
100
50
0
DMEM
LPS
LPS + L. gasseri (0.0001)
LPS + L. gasseri (0.001)
LPS + L. gasseri (0.01)
LPS + L. gasseri (0.1)
LPS + L. gasseri (1)
LPS + L. gasseri (2)
B
Cell viability (%)
DMEM
LPS
LPS + L. paracasei (0.0001)
LPS + L. paracasei (0.001)
LPS + L. paracasei (0.01)
LPS + L. paracasei (0.1)
LPS + L. paracasei (1)
LPS + L. paracasei (2)
C
Cell viability (%)
150
100
50
0
DMEM
LPS
LPS + L. casei (0.0001)
LPS + L. casei (0.001)
LPS + L. casei (0.01)
LPS + L. casei (0.1)
LPS + L. casei (1)
LPS +L. casei (2)
D
Cell viability (%)
DMEM
LPS
LPS + S. thermophilus (0.0001)
LPS + S. thermophilus (0.001)
LPS + S. thermophilus (0.01)
LPS + L. S. thermophilus (0.1)
LPS + S. thermophilus (1)
LPS + S. thermophilus (2)
E
Cell viability (%)
DMEM
LPS
LPS + B. lactis (0.0001)
LPS + B. lactis (0.001)
LPS + B. lactis (0.01)
LPS + B. lactis (0.1)
LPS + B. lactis (1)
LPS + B. lactis (2)

◂**Fig. 1** Cell viability in RAW-264.7 cells stimulated with LPS and treated with paraprobiotics in different doses (0.0001; 0.001; 0.01; 0.1; 1 and 2 g/ml doses). *L. gasseri* (**a**); *L. paracasei* (**b**); *L. casei* (**c**); *S. thermophilus* (**d**); *B. lactis* (**e**). Supernatants were collected 24 h after treatment**.** Data were expressed as mean ± SD. The experiments were performed in triplicate with four wells for each condition. $P < 0.05$ denoted statistical difference between groups. *Different from DMEM; #Different from LPS

All experiments were performed in triplicate and four wells were used for each condition. The reactive oxygen species (ROS) production, oxidative damage, and inflammatory parameters were measured as described below ("Cell viability: MTT assay", "ROS production", and "Myeloperoxidase activity", respectively).

## Cell Viability: MTT Assay

An MTT cell viability assay was performed in RAW-264.7 cells [24]. 100 µL of MTT (0.5 µg/ml) were added in each well and the cells were incubated for 3 h. After the incubation period, the MTT was removed and 150 µL of isopropyl alcohol was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader. The experiments were performed in triplicate with four wells for each condition. The results were expressed as the percentage of viable cells in comparison to the control group (DMEM—untreated cells).

Cell viability was expressed as a percentage (%), assuming that the DMEM group had 100% viable cells. Note that the DMEM group bar was considered to be 100% for all the analysis; the other groups were calculated in relation to this.

## ROS Production

Samples were incubated with the carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) probe for 40 min. After incubating at 37 °C for 24 h, fluorescence was measured at 485 nm (excitation) and 527 nm (emission) wavelengths on a microplate reader (Molecular Devices Spectra MAX M2, San José, Califórnia, EUA) [25].

## Oxidative Damage

Oxidative damage to proteins was examined by the quantifying carbonylated proteins. A reaction of carbonyl groups with dinitrophenylhydrazine in oxidized proteins, according to the method described by Levine et al. [26] was carried out. The absorbance was evaluated at 340 nm wavelengths on a microplate reader (Molecular Devices Spectra MAX M2, San José, Califórnia, EUA).

The formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction was measured as an index of oxidative stress as described previously [27]. The samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% (Sigma-Aldrich) and then heated in a boiling water bath for 15 min. Malondialdehyde (MDA) equivalents were determined by measuring the absorbance at 535 nm in SpectraMax Molecular Devices M2 (San José, Califórnia, EUA) using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) as an external standard. Results were expressed as MDA equivalents per mg of protein.

## Nitrosative Damage

Nitrite/nitrate concentration was assayed spectrophotometrically using Griess reagents (1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in bi-distilled H2O [NED solution]) and vanadium (III) chloride as previously described by Green et al. [28]. A standard curve was obtained simultaneously with each set of samples, and the optical density at 550 nm (OD550) was measured using an ELISA microplate reader (Molecular Devices Spectra MAX M2, San José, Califórnia, EUA).

## Myeloperoxidase Activity

The tissue was homogenized (50 mg/ml) in 0.5% of hexadecyltrimethylammonium bromide (Sigma-Aldrich) and centrifuged (8765×*g*) for 10 min. The suspension was sonicated and an aliquot of supernatant was mixed with a solution of 1.6 mmol/l 3,3′,5,5′-tetramethylbenzidine (TMB) and 1 mmol/l $H_2O_2$. The MPO activity was measured spectrophotometrically at 650 nm at 37 °C. The results were expressed as mU/mg protein [29].

## Levels of Cytokines

Concentrations of TNF-α, IL-10, and IL-6 were determined in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) on microplate reader (Molecular Devices Spectra MAX M2, San José, Califórnia, EUA) using commercial kits (R & D System, Mineápolis, Minnesota, EUA) [30]. Briefly, 96-well plates were sensitized with a specific monoclonal antibody incubated overnight. The plates were blocked with 1% albumin. Samples and/or standards were added to the plate. Specific detection antibodies were added and incubated for 2 h. Then, streptavidin peroxidase was added to the plate and tetramethylbenzidine (TMB) substrate solution was added. The reaction was stopped with the addition of 2 N hydrochloric acid solution (stop solution). At each stage, the plates were washed with wash buffer. Average detection: TNF-α (0.034–2.006); IL-10 (0.055–2.133), and IL-6 (0.046–2.550).

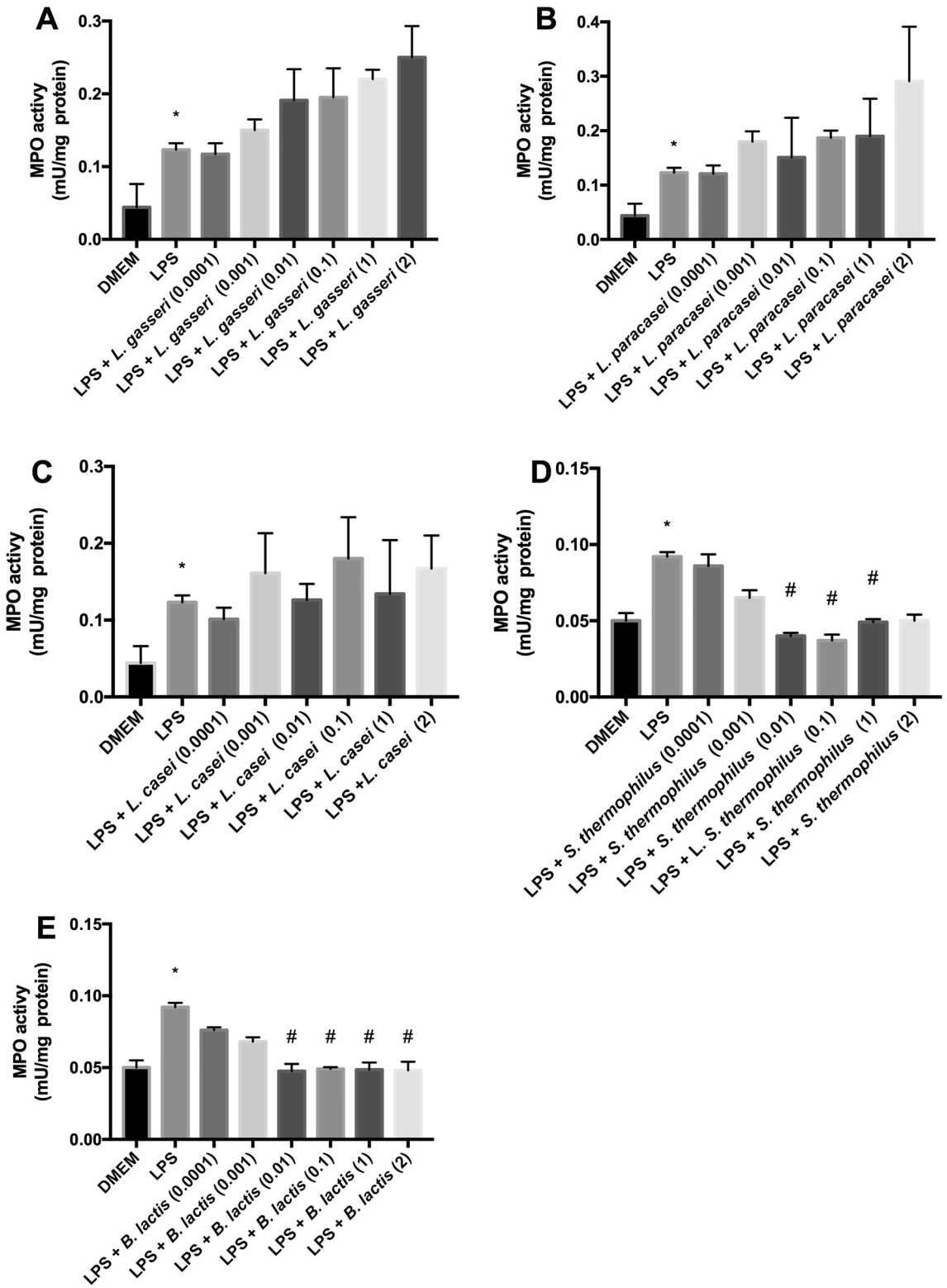



**Fig. 2** Mieloperoxidase activity in RAW-264.7 cells stimulated with LPS and treated with paraprobiotics in different doses (0.0001; 0.001; 0.01; 0.1; 1 and 2 g/ml doses). *L. gasseri* (**a**); *L. paracasei* (**b**); *L. casei* (**c**); *S. thermophilus* (**d**); *B. lactis* (**e**). Supernatants were collected 24 h after treatment. Data were expressed as mean ± SD. The experiments were performed in triplicate with four wells for each condition. $P < 0.05$ denoted statistical difference between groups. *Different from DMEM; #Different from LPS

### Statistical Analysis

Data collected were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey post hoc method and expressed as mean ± standard deviation in Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 21. Graphs were obtained using GraphPad Prism (San Diego, California, USA) version 7. For all comparisons, $P < 0.05$ indicated statistical significance.

## Results

The effects of paraprobiotics (MAMPs) on cell viability using the RAW-264.7 cell line were first analyzed. In the LPS group, there was a significant reduction in cell viability in relation to the control group. *L. gasseri, L. paracasei,* and *L. casei,* (Fig. 1a–c), 0.1, 1, and 2 g/ml improved cell viability when compared to the LPS group. In *S. thermophilus* (Fig. 1d), a protective effect from 0.01 g/ml dose and from 0.001 g/ml to *B. lactis* (Fig. 1e) was observed.

The effect of paraprobiotics (MAMPs) on myeloperoxidase activity was also evaluated (Fig. 2). As expected, The LPS group increased MPO activity compared to the DMEM group, and there was no significant effect of *L. gasseri, L. paracasei,* and *L. casei* (Fig. 2a–c). In contrast, *S. thermophilus* and *B. lactis* decreased MPO activity from 0.01 g/ml to higher doses (Fig. 2).

Nitrite/nitrate levels were significantly increased in the LPS group (Fig. 3). There was a decrease in nitrite/nitrate concentrations only in lower doses of *L. gasseri*, *L. paracasei*, and *L. casei* (0.0001; 0.001, and 0.01 g/ml), as opposed to *S. thermophilus* and *B. lactis* (Fig. 3).

Since these first results demonstrated a protective effect of different paraprobiotics consistently at 0.01 and 0.1 g/ml doses, cytokines (TNF, IL-6, and IL-10) and oxidative stress (DCF-DA, TBA, and Carbonyl) were also analyzed using these doses. Maltodextrin has been included in the next analyses; this compound is the vehicle used to produce MAMPs and we want to make sure that it does not influence the analysis (Fig. S1).

TNF levels increased in the LPS group, but paraprobiotics did not have any significant effect (Fig. 4a, b). IL-6 levels were increased in the LPS group when compared to the DMEM and maltodextrin group (Fig. 4c, d). There was a significant reduction in IL-6 levels at 0.1 g/ml dose in all analyzed paraprobiotics (Fig. 4d). In contrast to the 0.01 g/ml, a significant effect was only observed in *L. paracasei, B. lactis,* and *S. thermophilus* (Fig. 4c). Additionally, IL-10 levels decreased in the LPS group and increased only at the 0.1 g/ml dose in all paraprobiotics (Fig. 4e, f).

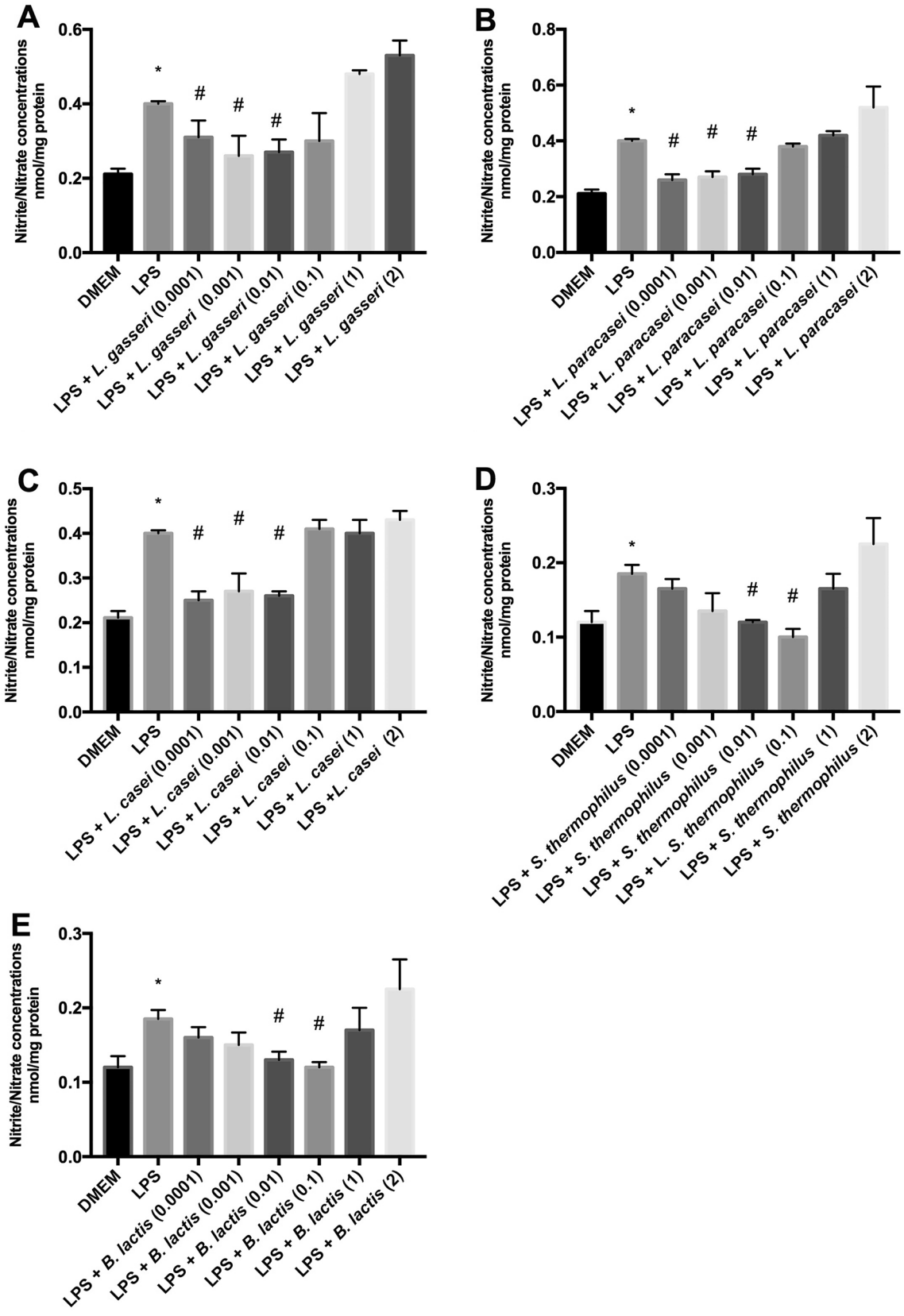
The effect of paraprobiotics (MAMPs) on ROS production was analyzed in RAW-264.7 cultures stimulated with LPS, using the fluorescent probe DCF-DA (Fig. 5). The ROS generation increased in the LPS group when compared with DMEM and maltodextrin, and *B. lactis* decreased ROS only at 0.01 g/ml dose, while *L. casei, L. paracasei, L. gasseri,* and *S. thermophilus* were effective at 0.1 g/ml dose (Fig. 5a, b). The DCF-DA results were reinforced by the observed in protein carbonyl and TBARS levels (Figs. 5e, f). The 0.1 g/ml dose was able to decrease protein carbonyl levels compared to LPS groups for all paraprobiotics (Fig. 5c, d).

The results are consistent with the protein damage measured by carbonyl assay. There was an increase in the LPS group, confirming oxidative stress, and a reversion is noted in the dose of 0.01 g/ml except *L. casei* and *B. lactis* in this dose and 0.1 g/ml for all paraprobiotics except *S. thermophilus*, which was less effective (Fig. 5c, d). Besides this, the images indicate that the cells preserved the structure in the control and paraprobiotics groups in LPS-induced RAW-264.7 cells in both doses (Figs. S2 and S3). In the LPS group, the cells had an altered form, indicating cell activation (Figs. S2 and S3).

## Discussion

The purpose of this study was to evaluate the effect of different paraprobiotics (MAMPs) on a lineage of RAW-264.7 macrophages stimulated with LPS. It is well known that LPS induces the activation of RAW-264.7 cells [31]; thus we used this paradigm to determine the anti-inflammatory effects of different paraprobiotics. LPS is a potent inducer of cytokines in monocytes, acting via the TLR4 receptor [32]. In addition, it occurs in nitric oxide production and MPO [33], which can induce oxidative stress. Thus, the protective effects of paraprobiotics demonstrated here suggest that they inhibit the LPS-TLR4 pathway, thus decreasing RAW-264.7 activation.

The inflammatory process must be modulated to provide a well-dimensioned defense mechanism. Inflammation is associated with a hemodynamic response [34, 35] and the dysregulation of inflammation plays an important role in different cellular dysfunctions [36, 37]. Studies indicate that paraprobiotics provide benefits such as the modulation of the immune system and secretion of metabolites by non-viable cells, in addition to adherence to intestinal cells that allows the inhibition of pathogens [2]. Experimental studies in neonate rats showed that MAMPs decreased LPS-induced pro-inflammatory and increased anti-inflammatory mediators [5]. In addition, emerging evidence indicates that strains of both *L. casei* [38–40] and *B. bifidum* [41–43] have beneficial effects in their heat-inactivated form through their anti-inflammatory and immunomodulatory effects [2]. A recent study published by Avila et al. [44] showed the beneficial effects of different probiotic


A
Nitrite/Nitrate concentrations nmol/mg protein
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DMEM
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LPS + L. gasseri (0.0001)
LPS + L. gasseri (0.001)
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LPS + S. thermophilus (0.001)
LPS + S. thermophilus (0.01)
LPS + L. S. thermophilus (0.1)
LPS + S. thermophilus (1)
LPS + S. thermophilus (2)
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DMEM
LPS
LPS + B. lactis (0.0001)
LPS + B. lactis (0.001)
LPS + B. lactis (0.01)
LPS + B. lactis (0.1)
LPS + B. lactis (1)
LPS + B. lactis (2)

◂**Fig. 3** Nitrite/Nitrate concentrations in RAW-264.7 cells stimulated with LPS and treated with paraprobiotics in different doses (0.0001; 0.001; 0.01; 0.1; 1 and 2 g/ml doses). *L. gasseri* (**a**); *L. paracasei* (**b**); *L. casei* (**c**); *S. thermophilus* (**d**); *B. lactis* (**e**). Supernatants were collected 24 h after treatment**.** Data were expressed as mean ± SD. The experiments were performed in triplicate with four wells for each condition. $P < 0.05$ denoted statistical difference between groups. *Different from DMEM; #Different from LPS

strains, including *L. casei*, in reducing the levels of nitrite/nitrate, MPO, and pro-inflammatory cytokines (IL-1, IL-6) in animals subjected to LPS-induced inflammation. In another study by Cross et al. [45], heat-inactivated *L. casei* did not induce IL-12 and TNF-α, when compared to viable *L. casei*, reinforcing the potential anti-inflammatory effect of paraprobiotics (MAMPs). In addition, Del Carmen et al. [46, 47] showed that *S. thermophilus* has intrinsic immunomodulatory properties, where expressing an antioxidant enzyme enhances its anti-inflammatory activities.

The microorganism inactivation process is fundamental to guaranteeing the immunomodulatory activity since such activity is mediated by all the structural components of the cells, as suggested by Tejada-Simon and Pestka [48]. The RAW-264.7 macrophages were exposed to heat-inactivated paraprobiotics, *Bifidobacterium* sp., *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus reuteri*, and *Streptococcus thermophilus*. Non-viable fragments of microbial origin have noticeable advantages over probiotics for industries and consumers in the development of safe and stable products. They have simple handling and can be used in products that go through different processes in the industry, a longer *Shelf life*, and are advantages in the use of paraprobiotics. These advantages allow the supply of these compounds to different consumers, areas, and industries, reducing the risk of microbial translocation and infection or even improving inflammatory responses in consumers with altered or compromised immune systems [49].

Oxidative stress results in protein denaturation, DNA hydroxylation, apoptosis and lipid peroxidation compromising the cells' viability [50, 51]. Many authors have reported that probiotics have antioxidant properties [52–55]. Several mechanisms are speculated, such stimulation of the immune system, neutralization of oxidants in the intestinal tract (by antioxidant enzymes) and the inhibition of intestinal pathogens [56].

The effect of paraprobiotics on intracellular ROS production in RAW-264.7 cultures was analyzed using the peroxide-sensitive DCF-DA probe, and the greatest effectiveness of MAMPs was shown in the dose of 0.1 g/ml for oxidative stress. It was found that ROS has extremely high reactivity, which gradually leads to oxidative damage to biomolecules [57]. In addition, the measurement of nitrate/nitrite concentration or total nitrate and nitrite concentration (NOx) is routinely used as an index of NO production [58] and unregulated production of nitric oxide can cause nitrosative stress, leading to damages of proteins/DNA and cell injury and death [59, 60]. Our results demonstrate that the 0.1 g/ml dose of paraprobiotics is more effective in stress oxidative reduction. *L. casei, L. gasseri*, and *L. paracasei*, interestingly, showed efficacy at much lower doses (0.0001, 0.001, and 0.01 g/ml).

It has already been established that Lactobacillus strains possess an antioxidant capacity [61]. Xing et al. [62] found that 13 tested Lactobacillus strains have an antioxidant effect on RAW-264.7 cells. Until now, there are many studies on the antioxidant activities of Lactobacillus still totally relying on the chemical way that do not consider the metabolism and bioavailability of antioxidants. Furthermore, in a recent study, Magistrelli et al., [63] showed that *Lactobacillus* strains exert promising results in decreasing oxidative stress, pro-inflammatory cytokines and potentially pathogenic bacterial overgrowth in the PBMC cells of patients with Parkinson's disease.

The differences in the mode of action of MAMPs of paraprobiotics verified in the present study, be it in the concentration or in the type of microorganism used, may be associated with the different structural components of cells that may be part of several bacteria, such as the constituents of the cell wall, which interact with immune cells in a specific way. Peptidoglycan is the main constituent of Gram-positive bacterial cell walls, accounting for up to 90% of its weight, while it constitutes only 15–20% of the cell wall in Gram-negative bacteria [2, 64]. On the walls of Gram-positive bacteria, there are molecules that project to the outer surface of the peptidoglycan layer, known as teicoic acids. These acids, together with proteins present on the cell wall surface, are responsible for the antigenic determination of Gram-positive bacteria because they differ between different species and lineages, where each combination and interaction with specific molecules and receptors can stimulate the body's immune activity in different ways. One advantage of using paraprobiotics is that this intervention may be safer than probiotics because they reduce the risk of infection, microbial translocation or enhanced inflammatory responses [14].

In general, our results demonstrate that 0.1 g/ml dose of paraprobiotics is more effective in reducing ROS generation and decreasing pro-inflammatory cytokines. *Lactobacillus* strains exert promising results, which may be due to the mode of action of each paraprobiotic, be it in the concentration or in the type of microorganism used, and may be associated with the different structural components of cells that may be part of several bacteria, such as the constituents of the cell wall, which interact with immune cells in a specific way. Paraprobiotics may contribute to improving intestinal

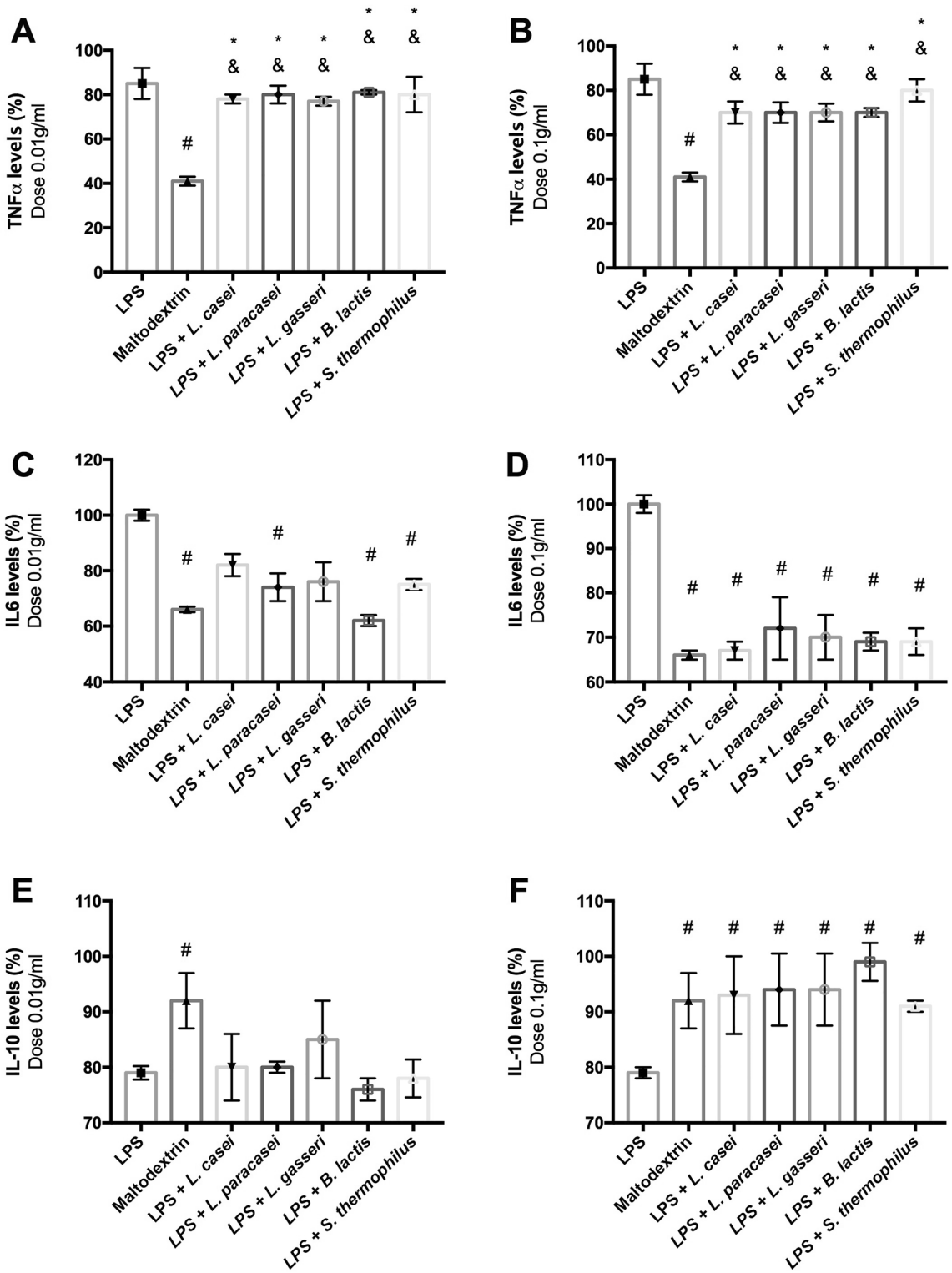


**Fig. 4** TNF levels (**a** and **b**); IL-6 levels (**c** and **d**); and IL-10 levels (**e** and **f**) in RAW-264.7 cells stimulated with LPS and treated with paraprobiotics in two different doses (0.01 and 0.1 g/ml doses). Supernatants were collected 24 h after treatment. Data were expressed as mean ± SD. The experiments were performed in triplicate with four wells for each condition. $P < 0.05$ denoted statistical difference between groups. *Different from DMEM; #Different from LPS; &Different from maltodextrin

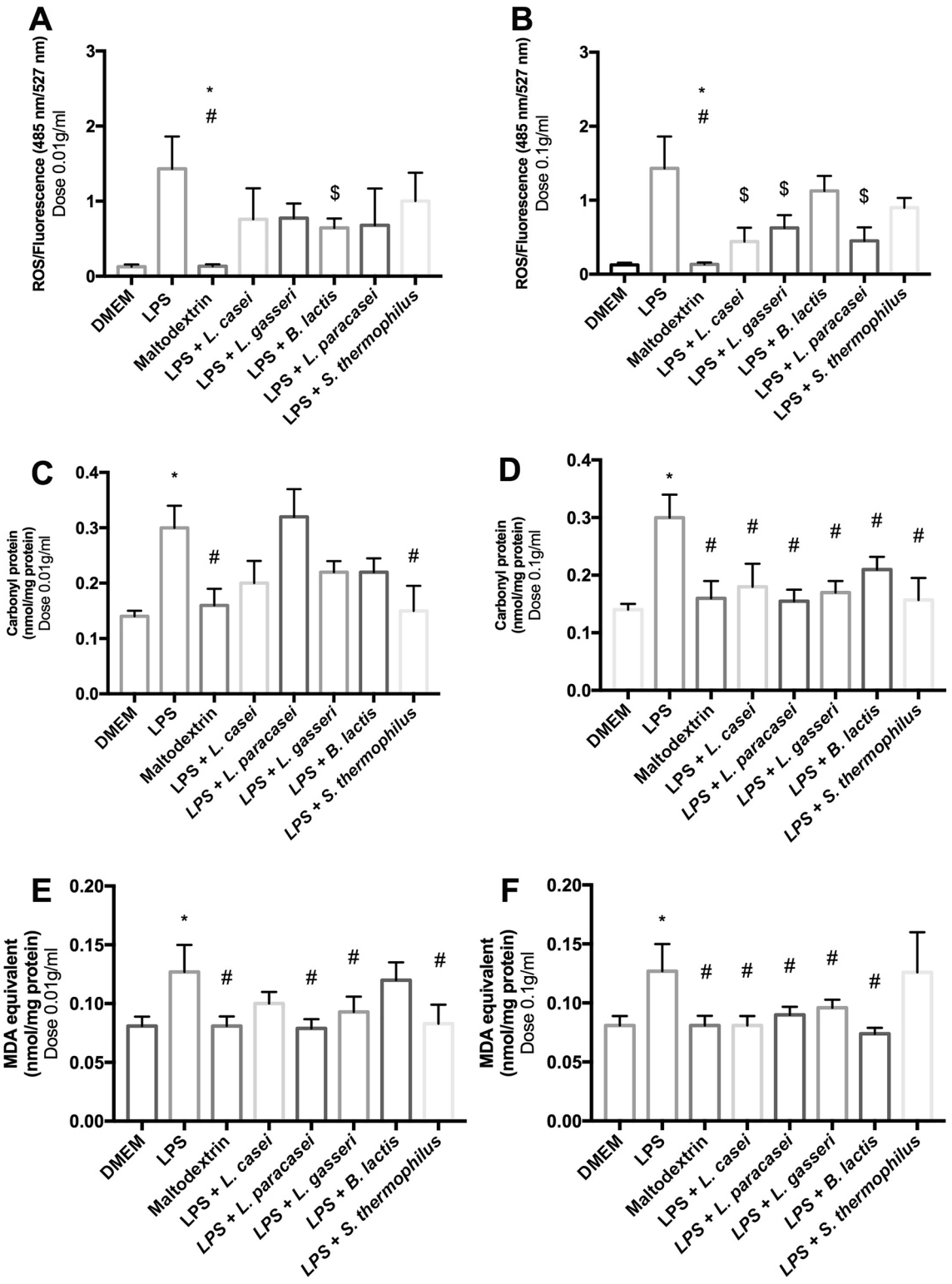


**Fig. 5** The levels of intracellular ROS were determined using DCF-DA (**a** and **b**), Carbonyl proteins (**c** and **d**), and MDA equivalents (**e** and **f**) in RAW-264.7 cells stimulated with LPS and treated with para-probiotics in two different doses (0.01 and 0.1 g/ml doses). All data are presented as mean ± SD. $n = 5$. *Different of DMEM; #Different of maltodextrin; $Different of LPS. $P < 0.05$

homeostasis, immunomodulation, and host metabolism. Thus, the use of paraprobiotics seems promising.

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## Declarations

**Conflict of interests** Gabbia Biotechnology is developing paraprobiotics for the commercial purposes. Gabriel Jesus, Marina Rosseto, and Ana Paula Voytena are members of Gabbia Biotechnology. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Consent to Publication** We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship, but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

**Data Availability** Data will be made available on reasonable request.

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