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Structural characterization and protective effect against murine sepsis of fucogalactans from *Agaricus bisporus* and *Lactarius rufus*

Andrea C. Ruthes[a], Yanna D. Rattmann[a], Elaine R. Carbonero[b], Philip A.J. Gorin[a], Marcello Iacomini[a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil
[b] Departamento de Química, Universidade Federal de Goiás, Campus Catalão, 75704-020 Catalão, GO, Brazil

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ABSTRACT

Fucogalactans from edible *Agaricus bisporus* (RFP-Ab) and wild *Lactarius rufus* (RFP-Lr) mushrooms were obtained on aqueous extraction followed by purification. RFP-Ab had M_w 43.8×10^4 g mol^{-1} and RFP-Lr M_w 1.4×10^4 g mol^{-1}. RFP-Lr had a (1 → 6)-linked α-D-Gal*p* main-chain partially substituted at O-2 by nonreducing end-units of α-L-Fuc*p* (29%). While RFP-Ab had a similar main chain, it was partially substituted at O-2 by nonreducing end-units of α-L-Fuc*p* (2.8%) and β-D-Gal*p* (14.5%), and partially methylated at HO-3. Both RFP-Lr and RFP-Ab were tested in mice against polymicrobial sepsis. Lethality rate, myeloperoxidase (MPO) activity and cytokine levels were determined. It was observed a reduction in late mortality rate by 62.5% and 50%, respectively, prevention of neutrophil accumulation in ileum and decreasing in TNF-α and IL-1β serum levels.

1. Introduction

Mushrooms have been used as therapeutic agents and valuable food sources for centuries in Asia, especially because of their chemical composition and nutritional values. They have high protein and fiber contents with small amounts of fat (Lakhanpal & Rana, 2005; Manzi, Aguzzi, & Pizzoferrato, 2001), and can be considered as functional foods, which can provide health benefits beyond the traditional nutrients they contain.

The practice of using hot-water extracts and decocts of mushrooms in oriental medicine, particularly in China, is the basis of modern studies on the medicinal properties. Approximately 300 species are known to have medicinal or nutraceutical properties and another 1800 have been identified, which have prospective medicinal properties (Lakhanpal & Rana, 2005).

From these, a great variety of active molecules, featuring immunomodulatory and antitumoral properties, reduction of blood pressure, among others (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007), have been identified in many mushrooms species. Among these, polysaccharides isolated from fruiting bodies, mycelia, and culture media (Zhang et al., 2007) deserve attention.

Several polysaccharides have been isolated from basidiomycetes, such as linear or branched glucans and heterogalactans, which can contain *O*-methyl groups or a variety of side chains (Wasser, 2002; Zhang et al., 2007). Most heterogalactans have a main chain composed of (1 → 6)-linked α-D-Gal*p* units with mainly fucose or mannose as substituents (Carbonero, Gracher, Komura, et al., 2008; Carbonero, Gracher, Rosa, et al., 2008; Rosado et al., 2003; Smiderle et al., 2008; Wasser, 2002; Zhang et al., 2007), although there are few studies relating their detailed structure and biological activity.

Many of these polysaccharides have been evaluated as biological response modifiers, especially for their antitumor activity (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser & Weis, 1999; Zhang et al., 2007). Some reports have shown that extracts from mushrooms can have other effects, such as anti-inflammatory and antinociceptive activity (Komura et al., 2010; Smiderle et al., 2008). However, it is not possible to attribute a relation between structure and activity, because most of the investigations were carried out using crude polysaccharide extracts (Lindequist, Niedermeyer, & Julich, 2005; Poucheret, Fons, & Rapior, 2006). Recent studies concerned anti-inflammatory and antinociceptive effects of fucogalactans, fucomannogalactans, and mannogalactans isolated from *Agaricus brasiliensis* and *Agaricus bisporus* var. *hortensis* (Komura et al., 2010), *Lentinus edodes* (Carbonero, Gracher, Komura, et al., 2008), and *Pleurotus pulmonarius* (Smiderle et al., 2008), respectively.

However, there are very few reports dealing with the ability of mushroom polysaccharides in reducing mortality caused by sepsis

* Corresponding author. Tel.: +55 41 3361 1655; fax: +55 41 3266 2042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

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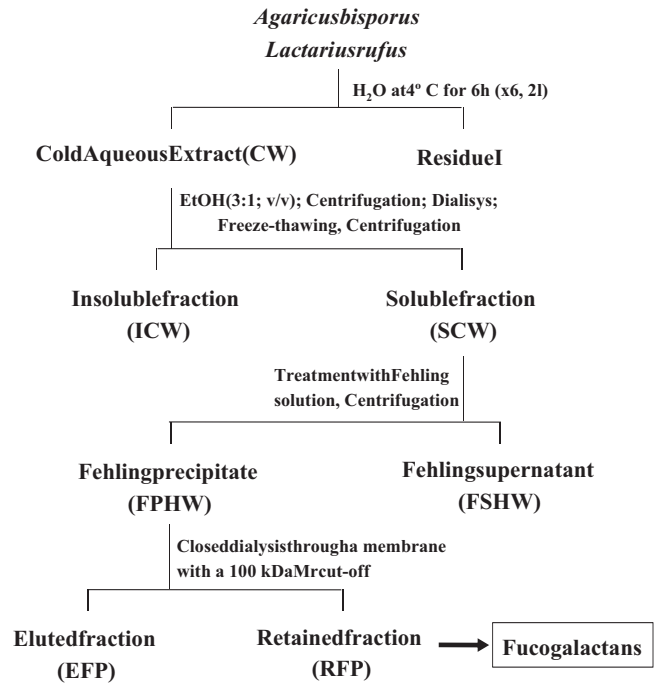

Fig. 1. Scheme of extraction and purification of fucogalactans from *A. bisporus* (Ab) and *L. rufus* (Lr).

in mice. Sepsis is a considerable health problem and a leading cause of morbidity and mortality in many intensive care units. It represents a state of overproduction of pro-inflammatory mediators which frequently occurs after various noxious injuries, especially bacterial infection, as a consequence of abdominal surgery, appendicitis, perforated ulcers, or an ischemic bowel (Angus et al., 2001).

Thus, in order to compare chemical structure with biological properties of mushroom polysaccharides, structures of fucogalactans isolated from the worldwide consumed mushroom *A. bisporus* (*champignon de Paris*; RFP-Ab), and from *Lactarius rufus* (RFP-Lr), which is used as condiment (Phillips, 2006), were now characterized. Their activities against murine sepsis, namely their effects on lethality, neutrophil migration and cytokine levels were also investigated.

2. Materials and methods

2.1. Biological materials

Dried fruiting bodies of *A. bisporus* (*champignon de Paris*) were provided by Makoto Yamashita Company (Miriam Harumi Yamashita), Sao José dos Pinhais, State of Paraná, Brazil. *L. rufus* fruit bodies were collected in middle of May, 2005 from soil of a *Pinus* sp. reforestation project located in Mafra, State of Santa Catarina, Brazil at latitude: 26°13′S; longitude: 49°50′W and altitude of 826 m above sea level. This mushroom sample was taken to the laboratory within 12 h of collection, cleaned up from forest debris (without washing) with a plastic knife, and dried in a freeze-dryer. Dried samples were then ground.

2.2. Extraction and purification of fucogalactans

Extraction of crude polysaccharides and their purification was carried out as in Fig. 1. Wiley-milled powder fruiting bodies from *A. bisporus* (100 g) and *L. rufus* (42 g) were extracted with H_2O at 4 °C for 6 h (6×; 2000 ml). The combined aq. extracts were added to excess ethanol (EtOH, 3:1; v/v) to precipitate polysaccharide, which was collected by centrifugation (8000 rpm at 5 °C for 20 min). Each sediment was then dissolved in H_2O, dialyzed against tap water for 20 h to remove low-molecular-weight carbohydrates, giving rise to solutions containing fractions CW-Ab and CW-Lr, respectively. These were frozen and then allowed to thaw slowly (Gorin & Iacomini, 1984), resulting in insoluble fractions ICW-Ab and ICW-Lr, which were separated by centrifugation as described above. The supernatant fractions SCW-Ab and SCW-Lr, were treated with Fehling solution (Jones & Stoodley, 1965), giving precipitated Cu^{2+} complexes (FP-Ab and FP-Lr, respectively), which were separated by centrifugation. The precipitates were neutralized with acetic acid (HOAc), dialyzed against tap water (48 h), deionized with mixed ion exchange resins, and then freeze-dried.

Each fraction was further purified by closed dialysis through a membrane with a 100 kDa M_r cut-off (Spectra/Por® Cellulose Ester), giving rise to an eluted (EFP) and a retained (RFP) material (Fig. 1).

2.3. Monosaccharide composition of polysaccharides

Each polysaccharide fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residues were successively reduced with $NaBH_4$ (1 mg) and acetylated with Ac_2O-pyridine (1:1, v/v; 200 μl) at 100 °C for 30 min following the method of Sassaki et al. (2008). The resulting alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS), using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, Model 810-R12 mass spectrometer. Incorporated was a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C min^{-1}, then hold, and the alditol acetates identified by their typical retention times and electron impact profiles.

2.4. Determination of homogeneity of polysaccharides and their molecular weight (M_w)

The homogeneity and molecular mass (M_w) of the purified fucogalactans RFP-Ab and RFP-Lr were determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector. The eluent was 0.1 M $NaNO_3$, containing 0.5 g l^{-1} NaN_3. The polysaccharide solutions were filtered through a membrane with 0.22 μm diameter pores (Millipore). The specific refractive index increment (dn/dc) was determined using a Waters 2410 detector, the samples being dissolved in the eluent, five increasing concentrations, ranging from 0.2 to 1.0 mg ml^{-1} being used to determine the slope of the increment.

2.5. Methylation analysis of fucogalactans

Per-*O*-methylation of each isolated polysaccharides (RFP-Lr and RFP-Ab; 10 mg) was carried out using NaOH–Me_2SO–MeI (Ciucanu & Kerek, 1984). The process, after isolation of the products by neutralization (HOAc), dialysis, and evaporation was repeated, and the methylation was found to be complete. The per-*O*-methylated derivatives were hydrolyzed with 45% aqueous formic acid (HCO_2H, 1 ml) for 6 h at 100 °C, followed by NaB_2H_4 reduction and acetylation as above (Section 2.3), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS using a Varian model 4000 gas chromatograph, equipped with fused silica capillary columns of CP-Sil-43CB. The injector temperature was maintained at 220 °C, with the oven starting at 50 °C (hold 2 min) to 90 °C (20 °C min^{-1}, then held for 1 min), 180 °C (5 °C min^{-1}, then held for 2 min) and to 220 °C (3 °C min^{-1}, then held for 5 min). Helium was used as carrier gas at a flow rate of 1.0 ml min^{-1}. Partially *O*-methylated alditol acetates were identified from m/z of their positive ions, by comparison with standards, the results being

expressed as a relative percentage of each component (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.6. Nuclear magnetic resonance (NMR) spectroscopy

Mono- (^{13}C, ^{1}H and DEPT) and bidimensional NMR spectra (HMQC, COSY, and coupled HMQC) were prepared using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Analyses were carried out at 70 °C on samples dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) and 2.21 (^{1}H), based on DSS (2,2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt; $\delta = 0.0$ for ^{13}C and ^{1}H).

2.7. Experimental animals

Male albino Swiss mice (3 months old, weighing 25–30 g), from the Universidade Federal do Paraná colony were used for biological tests. They were maintained under standard laboratory conditions, with a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C), Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available *ad libitum*. All experimental procedures were previously approved by the Institutional Ethics Committee of the University (authorization number 529).

2.8. Procedure to induce sepsis by cecal ligation and puncture (CLP)

Mice were randomly divided into five groups with 10 mice/group: sham-operation, CLP plus saline (10 ml kg^{-1} s.c.), CLP plus RFP-Lr (30 mg kg^{-1} s.c.), CLP plus RFP-Ab (30 mg kg^{-1} s.c.) and CLP plus dexamethasone (0.5 mg kg^{-1} s.c.). Saline was used as vehicle for dissolving both polysaccharides; and dexamethasone was commercially purchased. It was administered 60 μl of each treatment solution, regarding the corporeal weigh of the animals (~30 g). Ketamine (80 mg kg^{-1}) and xylazine (20 mg kg^{-1}) were injected intraperitoneally to anesthetize the mice before the surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision about ~1.5 cm was performed on the abdomen. The cecum was carefully isolated and the distal 50% was ligated. The cecum was then punctured twice with a sterile 20-gauge needle and squeezed to extrude the fecal material from the wounds. The cecum was replaced and the abdomen was closed in two layers. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received subcutaneous sterile saline injection (1 ml) for fluid resuscitation after surgery. The mice were then placed on a heating pad until they recovered from the anesthesia. Food and water *ad libitum* were provided throughout the experiment. Survival was monitored for 7 days, each 12 h. During this period, saline and drugs were subcutaneously administered daily.

In other experiments, during the surgery, mice were treated subcutaneously with saline, RFP-Lr, RFP-Ab or dexamethasone. After 18 h post-operation, mice were sacrificed. Their tissues from small intestines (ileum) and blood were collected and frozen for later use to determine the myeloperoxidase (MPO) activity and investigate cytokine production in serum.

2.9. Ileum MPO activity

MPO activity, assessment measure of neutrophil influx, was determined according to established protocols (Bradley, Priebat, Christensen, & Rothstein, 1982). Briefly, ileum tissue was homogenized in 0.5 ml of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 14,000 rpm at 4 °C for 15 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer (9.6 mM 3,3,5,5-tetramethylbenzine, 150 nmol l^{-1} H_2O_2 in 50 mM potassium phosphate buffer), and read at 620 nm. Results are expressed as change in optical density per milligram of protein (measured by Bradford assay).

2.10. Determination of cytokines levels

Tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β concentrations were determined in mice serum using enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.11. Statistical analysis

Data were expressed as means ± SE of five or ten mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Bonferroni's. Calculations were performed with Graphpad Prism 5.0. p values < 0.05 were considered significant.

3. Results and discussion

3.1. Fucogalactans structural characterization

Agaricus is an important fungal genus, considering that some of its species contain polysaccharides that have been presenting several biological activities, especially antitumor. The specie *A. bisporus* is one of the most extensively cultivated and consumed mushrooms in the world. In the other hand, *Lactarius* spp., such as *L. rufus*, which consumption is limited to its use as a condiment in a few countries, have not been studied in this context. In order to obtain pure heteropolysaccharides from the fruiting bodies of *A. bisporus* and *L. rufus*, each was submitted to aqueous extraction at 4 °C. The extracted polysaccharides were precipitated with excess ethanol, obtained as sediments on ultracentrifugation, dialyzed against tap water 4, and the solution freeze-dried to give CW-Ab and CW-Lr, respectively (Fig. 1).

Fractionation and purification of CW-Ab and CW-Lr was carried out by a freeze–thawing procedure (Gorin & Iacomini, 1984), resulting in a respective cold water-soluble SCW-Ab (4.2 g) and SCW-Lr (2.4 g) and discarded insoluble fractions.

Fraction SCW-Lr contained fucose, xylose, galactose and glucose, while SCW-Ab had in addition, 3-*O*-methyl-galactose (confirmed by the presence of the fragments with m/z 130 and 190 from derived mono-deuterated alditol acetates).

Fractions SCW-Ab and SCW-Lr gave heterogeneous HPSEC elution profiles, so they were then treated with Fehling solution. Their respective insoluble Cu^{2+} complexes FP-Ab (2.1 g) and FP-Lr (1.9 g) were further purified by closed dialysis through a 100 kDa M_r cut-off membrane. This procedure gave rise to eluted EFP-Ab and EFP-Lr and retained RFP-Ab and RFP-Lr fractions, respectively (Fig. 1). The RFP fractions (RPF-Ab: 205 mg and RPF-Lr: 301 mg) were homogeneous on HPSEC, and had M_w 43.8×10^4 g mol^{-1} (dn/dc 0.145 ml g^{-1}) for *A. bisporus* and M_w 1.4×10^4 g mol^{-1} (dn/dc 0.151 ml g^{-1}) for *L. rufus* (Fig. 2A and B, respectively).

Fraction RFP-Ab contained fucose (6%), galactose (84%) and 3-*O*-methyl-galactose (10%), whereas RFP-Lr, only contained fucose (29%) and galactose (71%).

Both fractions, RFP-Ab and RFP-Lr were submitted to methylation (Ciucanu & Kerek, 1984), where GC–MS of resulting *O*-methylalditol acetates showed the presence of branched structures, containing mainly, nonreducing end-units of Fuc*p* (2,3,4-Me_3Fuc), besides the 6-*O*-(2,3,4-Me_3Gal) and 2,6-di-*O*-substituted units (3,4-Me_2Gal) of galactopyranose. Nonreducing end-units of Gal*p* (2,3,4,6-Me_4Gal) were only detected in the

Table 1
Partially *O*-methylated acetates formed on methylation analysis of the fucogalactans obtained from *L. rufus* (Lr) and *A. bisporus* (Ab) fruiting bodies: linkage types.

Partially *O*-methylated alditol acetates[a]	*Rt*[b]	% Area of fragments[c]		Linkage type[d]
		RFP-Ab	RFP-Lr	
2,3,4-Me_3-Fuc	20.03	2.8	26.9	Fuc*p*-(1→
2,3,4,6-Me_4-Gal	21.19	14.5	–	Gal*p*-(1→
2,3,4-Me_3-Gal	23.67	64.8	46.0	6→)-Gal*p*-(1→
3,4-Me_2-Gal	25.76	17.9	27.1	2,6→)-Gal*p*-(1→

[a] GC–MS analysis on a CP-Sil-43CB capillary column.
[b] Retention time (min).
[c] Based on derived *O*-methylalditol acetates.
[d] Trace.

RFP-Ab isolated from *A. bisporus* (Table 1). The ratio of units of Fuc*p*:2,6-di-*O*-Gal*p*:6-*O*-Gal*p* was ~1:0.9:1.5 and 0.1:1:5 for RFP-Lr from *L. rufus* and RFP-Ab from *A. bisporus*, respectively, showing the major structural difference existent when comparing the two isolated fucogalactanas. These values were confirmed by the integration of H-1 signals (5.10:5.08:5.01) present in the ^{1}H NMR spectrum (data not shown).

Further NMR spectroscopy [^{1}H, ^{13}C and DEPT (Fig. 3), HMQC (Fig. 4), COSY and coupled HMQC (data not shown)] was also helpful in elucidating the fucogalactan structures (Table 2).

RFP-Lr ^{13}C NMR (Fig. 3A) and HMQC spectra (Fig. 4A), obtained using D_2O as solvent, had signals (C-1/H-1) at δ 103.9/5.10, 101.2/5.08, 100.9/5.08 and 100.7/4.98 corresponding to Fuc*p* units, 2,6-di-*O*- and 6-*O*-subst. Gal*p* units, respectively. While those from RFP-Ab (Figs. 3B and 4B, respectively), had C-1/H-1 signals at δ 103.9/5.10, 100.7/5.05 and 100.6/5.01 corresponding to Fuc*p* units, 2,6-di-*O*- and 6-*O*-subst. Gal*p* units, respectively. Anomeric signals corresponding to non-reducing end groups of β-Gal*p* (δ 105.5/4.64) and units of 3-Me-β-Gal*p* (δ 100.6/5.01) were present (Figs. 3B and 4B), in agreement with the methylation data

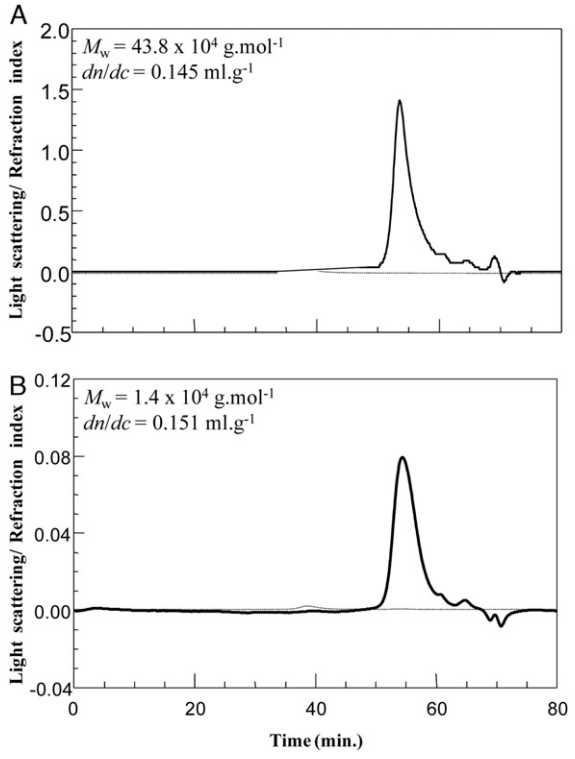

Fig. 2. Elution profiles of fractions RFP-Ab (B) and RFP-Lr (C) determined by HPSEC using light scattering (- - -) and refractive index detectors (—).

indicating the presence of nonreducing end-units of β-Gal*p* (Table 2). The glycosidic configurations of RFP-Lr and RFP-Ab fucogalactans units were confirmed by the values of the coupling constants $J_{C\text{-}1/H\text{-}1}$ found in ^{1}H/^{13}C-coupled HMQC spectra. The nonreducing end- of Fuc*p* and the main-chain units (galactose or 3-*O*-Me-galactose or both) had the α-configuration due to respective $J_{C\text{-}1/H\text{-}1}$ 162.6 Hz and 172.6 Hz, respectively, while those of nonreducing end-units of Gal*p* from RFP-Ab had β-configuration, consistent with $J_{C\text{-}1/H\text{-}1}$ 161.3 Hz (Perlin & Casu, 1969).

The above methylation analysis indicated the presence of 6-*O*- and 2-*O*-subst. Gal*p* units (Table 2), which was confirmed by NMR spectroscopy. *O*-subst. C-2 signals were at δ 82.8 and 81.6 for RFP-Lr and RFP-Ab, respectively (Figs. 3 and 4), and substituted –CH_2 groups of the 6-*O*-(Gal*p* e 3-*O*-Me-Gal*p*) and 2,6-di-*O*-subst. (Gal*p*) units of the main chain were at δ 69.4/69.7 and 70.0, respectively, giving rise to inverted signals in the DEPT spectra (Fig. 3A′ and B′). The presence and position of *O*-methyl groups of the heteropolysaccharide from *A. bisporus* were confirmed by δ 58.9/3.43 and δ 81.6/3.58 (C/H) signals corresponding to –OCH_3 and *O*-subst. C-3 substituted/H-3, respectively (Figs. 3B and 4B and Table 2).

The signals of C-2/H-2 to C-6/H-6 at δ 72.0/3.90, 71.2/3.85, 74.5/73.0/3.95, 69.9/4.11 and δ 18.2/1.27 corresponded to Fuc*p* units, while those at δ 70.1/3.88, 81.6/3.58, 68.2/4.30, 71.7/4.21 and δ 69.4/69.7/3.72/3.93 were from 3-*O*-Me-Gal*p* units from RFP-Ab fucogalactan (Figs. 3 and 4 and Table 2). Due to the presence of β-Gal*p* (14.5%) of the polymer isolated from *A. bisporus*, the signals of C-2 (δ 73.6), C-3 (δ 77.6), C-4 (δ 71.1), C-5 (δ 78.4) and C-6 (δ 63.6) were enhanced in its DEPT spectrum (Fig. 2B′).

Analysis of the fucogalactan RFP-Lr from *L. rufus* showed it to consist of a (1 → 6)-linked α-D-galactopyranosyl main-chain, partially substituted at O-2 by nonreducing end-units of α-L-Fuc*p*. That of the RFP-Ab from *A. bisporus* was related, although the main chain was partially *O*-methylated and had some β-D-Gal*p* nonreducing end-units as substituents.

Fucogalactans similar to those now described have been isolated from cultivated mycelium of *Coprinus comatus* (Fan et al., 2006), fruiting bodies of *Hericium erinaceus* (Zhang et al., 2006), *A. brasiliensis* and *A. bisporus* var. *hortensis* (Komura et al., 2010). However, RFP-Lr had greater fucose content among the fucogalactans already characterized, while RFP-Ab had more nonreducing end-units of β-Gal*p* than the fucogalactan from the related species *A. bisporus* var. *hortensis* (Komura et al., 2010).

3.2. Biological experiments

It is generally accepted that polysaccharides isolated from mushrooms and plants enhance various immune responses *in vivo* and *in vitro*. There are also many reports of mushroom polysaccharide-induced nonspecific resistance against diverse microbial pathogens.

In order to determine effects on murine sepsis of the fucogalactans RFP-Ab and RFP-Lr, isolated from *A. bisporus* and *L. rufus*, respectively, they were tested at doses of 30 mg kg^{-1}. Thus their effects on the survival rate of infected mice, neutrophil influx, and cytokine levels were determined.

The results of the survival experiments are shown in Fig. 5A. Mice treated with saline started to die between 12 h and 24 h after CLP, with a death rate reaching 37.5% and 87.5% at 24 h and 72 h post-CLP, respectively. The overall mortality at the end of the observation period was 100%. The lethality was markedly delayed in mice treated with both fucogalactans, RFP-Ab and RFP-Lr. At 24 h and 72 h after CLP, the mortality rate in RFP-Lr group was zero and 25%, respectively. At the end of the study, the overall survival in this group was 62.5%. As for RFP-Ab, at 24 h and 72 h after CLP, their mortality rate was zero and 37.5%, respectively, with an overall survival of 50.0% (Fig. 5A). Dexamethasone-treated mice showed a

Table 2
^{1}H and ^{13}C NMR chemical shifts [expressed as δ (ppm)] of the fucogalactans from *A. bisporus* (RFP-Ab) and *L. rufus* (RFP-Lr).[a]

Units		1	2	3	4	5	6		-*O*-CH_3
							a	*b*	
α-L-Fuc*p*-(1→	^{13}C	103.9	72.0	71.2	74.5/73.0	69.9	18.2	–	–
	^{1}H	5.10	3.85	3.89	3.85	4.20	1.27	–	–
β-D-Gal*p*-(1→	^{13}C	105.5	73.6	77.6	71.1	78.4	63.6	–	–
	^{1}H	4.64	3.85	3.84	4.08	3.58	3.80	–	–
2,6→)-α-D-Gal*p*-(1→	^{13}C	101.2/100.7	82.8/79.4	71.1	72.2	71.7	69.7	69.7	–
	^{1}H	5.08	3.61/3.84	4.08	4.09	4.21	3.70	4.01	–
6→)-α-D-Gal*p*-(1→	^{13}C	100.7	71.1	72.4	72.4	71.7	69.3	69.3	–
	^{1}H	5.01	3.84	3.89	4.03	4.21	3.72	3.93	–
6→)-3-O-Me-α-D-Gal*p*-(1→	^{13}C	100.6	70.1	81.6	68.2	71.7	69.4	69.4	58.9
	^{1}H	5.01	3.88	3.58	4.3	4.21	3.89	3.93	3.43

[a] Assignments are based on ^{13}C, ^{1}H, DEPT, COSY and HMQC examination.

significant improvement in survival, with an overall survival rate of 16.7% at the end of the observation period. However, this anti-inflammatory medicine significantly delayed the onset of death after CLP (~36 h versus saline group). No death was observed in the sham-operated mice.

In terms of neutrophil influx, it was inhibited by polysaccharide compounds. CLP surgery markedly increased ileum tissue MPO levels compared with sham group (42.3%) (Fig. 5B). This increase in tissue MPO was significantly prevented by both polysaccharides RFP-Lr and RFP-Ab, with an inhibition of 43.0% and 51.9%, respectively, versus saline group (Fig. 5B). Dexamethasone, the anti-inflammatory control, strongly inhibited the MPO activity in ileum (61.2%).

TNF-α and IL-1β cytokines levels were lower in sham surgery control mice. In contrast, 18 h after CLP surgery, both showed an increase of 49% and 48%, respectively, in comparison to the sham group (Fig. 6A and B). The treatment with both fucogalactans showed significant reduction in cytokine production in relation to saline group. RFP-Lr reduced the levels of TNF-α in 33.6% and 38.8% of IL-1β, while RFP-Ab reduced 36.5% and 32.3%, respectively. The increased levels of TNF-α and IL-1β were significantly prevented by dexamethasone (0.5 mg kg^{-1}), which promoted an inhibition of

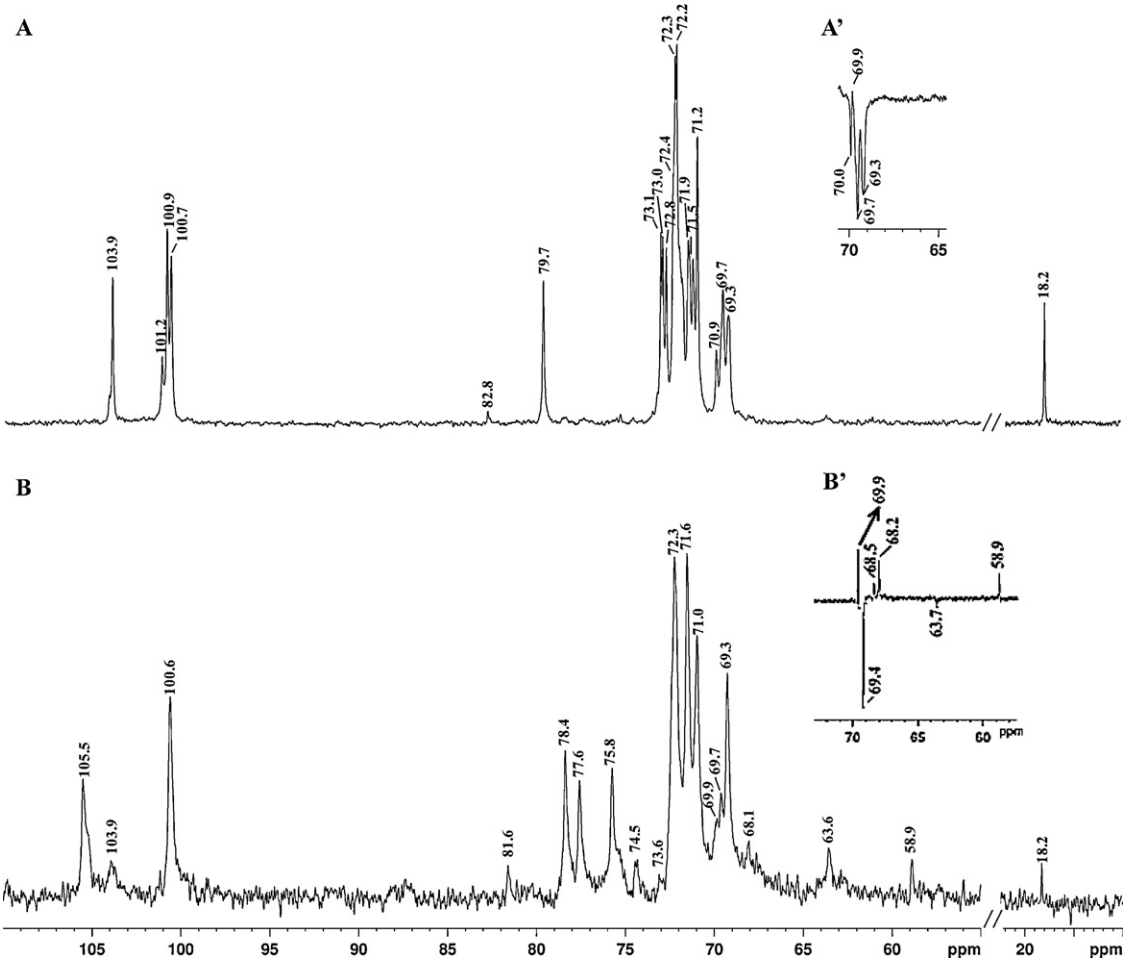

Fig. 3. ^{13}C NMR spectra of fucogalactan fractions RFP-Lr (A) and RFP-Ab (B) with inserts of DEPT –CH_2 inversion (A′ and B′, respectively).

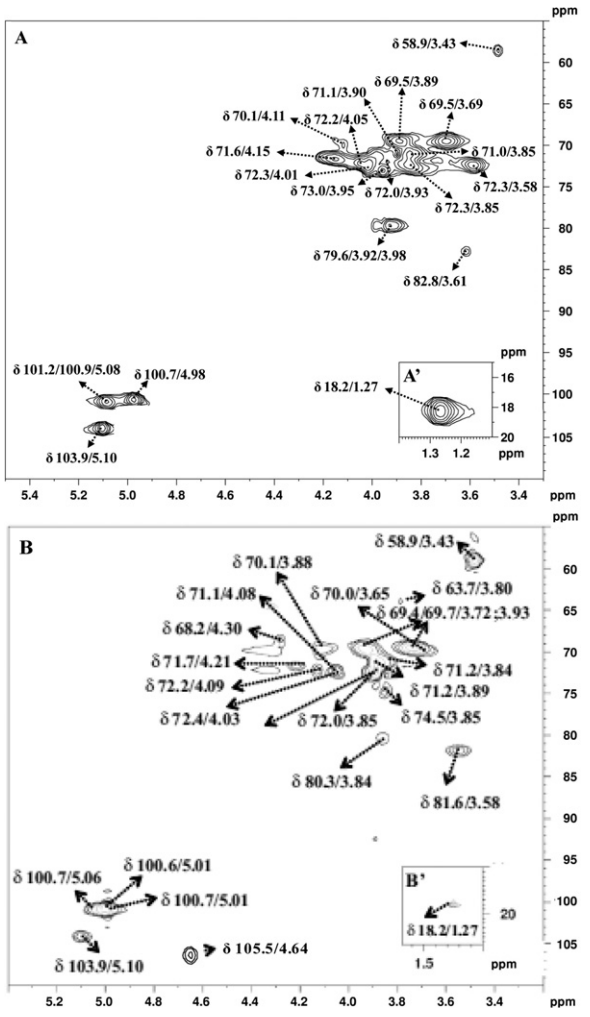

Fig. 4. ^{1}H (obs.)/^{13}C HMQC spectra of RFP-Lr (A) and RFP-Ab (B) of fucogalactans fractions (chemical shifts are expressed in δ ppm).

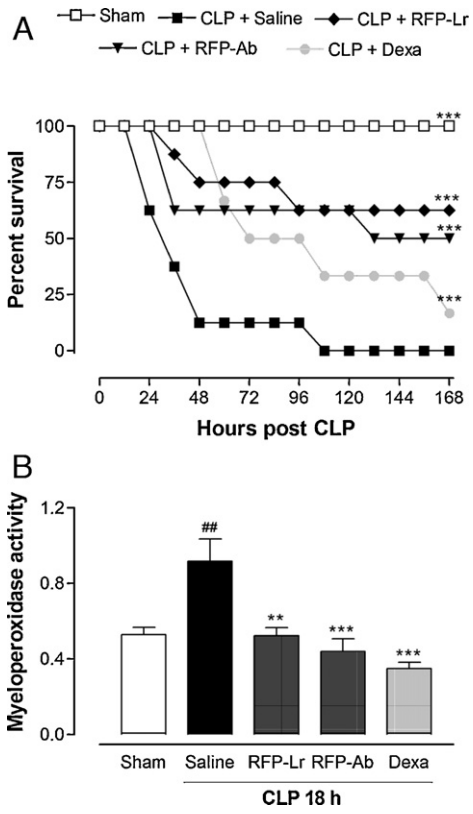

Fig. 5. Effects of the polysaccharides RFP-Lr and RFP-Ab on survival rate and on myeloperoxidase activity in sepsis. (A) Mice were randomly divided into five groups. All groups except the sham group received cecal ligation and puncture (CLP) surgery. Saline, RFP-Lr (30 mg kg^{-1} s.c.), RFP-Ab (30 mg kg^{-1} s.c.) and dexamethasone (0.5 mg kg^{-1} s.c.) were administered immediately after surgery. The treatment continued over the next days, each 24 h. The survival rate of mice was recorded every 12 h over 7 days after CLP. (B) Mice subjected to sepsis by CLP were treated with saline, RFP-Lr, RFP-Ab or dexamethasone immediately after surgery. The myeloperoxidase activity (MPO, indicator of neutrophil accumulation) was measured spectrophotometrically in ileum samples harvested 18 h after the induction of CLP. Results were expressed as change in optical density per milligram of protein. Values represent means ± SEM. $^{***}p < 0.001$ and $^{**}p < 0.01$, indicated value versus CLP plus saline group; $^{\#\#}p < 0.01$, CLP plus saline versus sham. ANOVA followed by Bonferroni's test.

approximately 42%, in both cases, in comparison with saline group (Fig. 6, panels A and B).

It is believed that sepsis may lead to aberrant host inflammatory responses, causing cell injury and organ dysfunction. During sepsis, the intestine is considered not only to be a source of bacteremia but also an important target of bacterial products with major functional consequences for intestinal motility and the generation of inflammatory mediators and recruitment of immune cells. Neutrophil infiltration is an important pathophysiologic alteration associated with sepsis. These cells directly damage tissue by releasing pro-inflammatory mediators, as cytokines, superoxide-derived free radicals and lysosomal enzymes, such as MPO, which amplify the systemic inflammatory response and cause multiple organ failure (Landry & Oliver, 2001; Mainous, Ertel, Chaudry, & Deitch, 1995).

In this study, a polymicrobial sepsis was induced by cecal ligation and puncture (CLP) in mice to investigate the effects of fucogalactans RFP-Lr and RFL-Ab. This model mimics the sepsis in human, caused by pathogens derived from the intestinal tract, and is considered to closely simulate clinical situation (Otero-Anton et al., 2001). Lethality was markedly delayed in mice receiving both polysaccharides (see Fig. 5A). This result could be attributable to an anti-inflammatory activity, which is consistent with previous literature findings (Ahn et al., 2006; Kim et al., 2003; Komura et al., 2010). There are many reports of mushroom polysaccharides inducing nonspecific resistance against diverse microbial pathogens in animals (Cisneros, Gibson, & Tzianabos, 1996; Kernodle, Gates, & Kaiser, 1998; Tzianabos & Cisneros, 1996), as well as in human (Babineau, Hackford, et al., 1994; Babineau, Marcello, et al., 1994) models. Although the mechanism that mushroom polysaccharides induced effects is not well understood, a key host immune response modulation caused by these mushroom polymers appears to be central (Wasser, 2010). In addition, a study carried out on experimental sepsis showed that a pretreatment with an acidic polysaccharide isolated from the medicinal mushroom *Phellinus linteus* could be able to inhibit bacterial septic shock by suppressing TNF-α production in areas of inflammation, suggesting that the mushroom polysaccharide may contribute to prolong the survival of septic shock mice (Kim et al., 2003). A similar result was observed at the present work, where both fucogalactans tested were able to decrease significantly TNF-α and also IL-1β serum levels. Several previous studies have demonstrated that some cytokines, especially TNF-α, IL-1β and IL-6 are strongly associated with sepsis syndrome, therefore inhibiting the pro-inflammatory cytokine overproduction during early sepsis may reduce sepsis outcome (Cohen, 2002; Ebong et al., 1999; Takala, Nupponen, Kylanpaa-Back, & Repo, 2002). Consequently, the reduction in mice lethality caused by both fucogalactans may be also related to the decreasing in TNF-α and IL-1β serum levels.

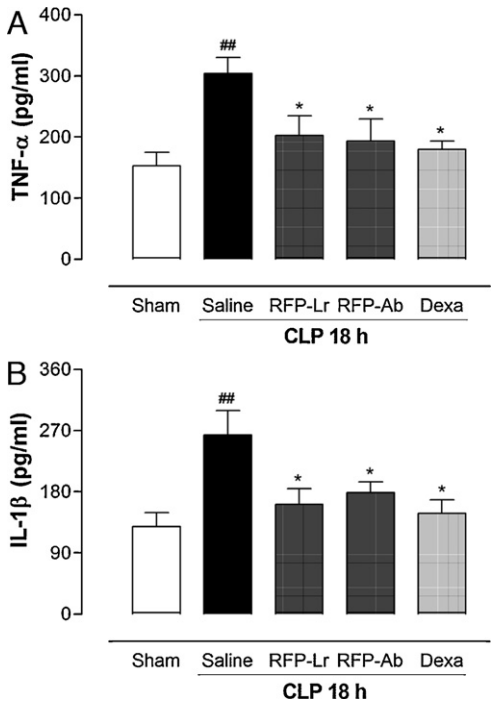

Fig. 6. Effect of fucogalactans RFP-Lr and RFP-Ab upon TNF-α (A) and IL-1β (B) production in infected mice serum. The mice, except the sham group, received sterile saline, RFP-Lr (30 mg kg^{-1} s.c.), RFP-Ab (30 mg kg^{-1} s.c.) or dexamethasone (0.5 mg kg^{-1} s.c.), which were administered during the CLP-surgery, and the cytokines levels were evaluated 18 h after onset. Each group represents the mean ± SEM of four to five animals. $^{*}p < 0.05$, indicated value versus CLP plus saline group; $^{\#\#}p < 0.01$, CLP plus saline versus sham. ANOVA followed by Bonferroni's test. A false-operated group (sham) was also provided for this test.

Since MPO is a lysosomal enzyme of polymorphonuclear leukocytes that acts as a catalyst in the production of hypochlorous acid (powerful oxidant), the effects of RFP-Lr and RFP-Ab on MPO activity were also investigated. RFP-Lr and RFP-Ab could prevent the elevation of MPO activity, indirectly indicating reductions in neutrophil recruitment to ileum, and also in oxidative tissue damage (Fig. 5B). This is of particular relevance because oxidative stress is a likely mechanism for gut mucosal barrier dysfunction in sepsis, a condition that can amplify and perpetuate the initial systemic inflammatory response (Pastores, Katz, & Kvetan, 1996).

Therefore, one can suggest that *A. bisporus* and *L. rufus* fucogalactans have an anti-inflammatory action, such as those from *A. brasiliensis* and *A. bisporus* var. *hortensis* (Komura et al., 2010). So this biological effect may be related to their structures, since it was observed influence of structure on biological response when similar polymers, having small differences in the main chain, such as the presence of *O*-methyl groups, or more significant ones in the side chains, were evaluated. Based on the fucogalactans, one can suggest that factors like M_w and chemical structure could be significant. RFP-Ab had a greater M_w than RFP-Lr, while both fucogalactans contained a (1 → 6)-linked α-D-galactopyranosyl main-chain, partially substituted at O-2, RFP-Ab, besides lower amounts of α-L-Fuc*p*, had β-D-Gal*p* as nonreducing end-units, the latter being absent in RFP-Lr. Consequently, determination of the polysaccharides fine structure is significant in understanding the relation between their structure and biological activity.

In summary, fucogalactans from edible *A. bisporus* (RFP-Ab) and wild *L. rufus* (RFP-Lr) mushrooms were purified and their structures determined. They could prevent the lethality caused by polymicrobial sepsis in mice. This beneficial effect seems to be, at least in part, due to a reduction in neutrophil migration (MPO essay), decreasing of circulating pro-inflammatory cytokines and consequent protection against organ damage, although further investigation is required to clarify the detailed mechanism.

4. Conclusions

The fucogalactans from *A. bisporus* and *L. rufus* have an anti-inflammatory action, such as those from *A. brasiliensis* and *A. bisporus* var. *hortensis* (Komura et al., 2010), related to their structures. The influence of structure on the biological response was found with similar polymers, having small differences in the main chain, such as the presence of *O*-methyl groups, or more significant ones in the side chains. Consequently, determination of the fine structure of the polysaccharides is significant in understanding the relation between their structure and biological activity. To determine their mechanism of action, other biological assays are necessary.

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