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Oral administration of an aqueous extract from the oyster mushroom *Pleurotus ostreatus* enhances the immunonutritional recovery of malnourished mice

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Abstract

Mushroom nutriceutical components have lately attracted interest for developing immunonutritional support. However, there is relatively little information pertaining to the use of mushroom preparations for modulating the metabolic and immunological disorders associated to malnutrition. This study was aimed to evaluate the effects of oral administration of an aqueous extract (CW-P) from *Pleurotus ostreatus* on the recovery of biochemical and immunological functions of malnourished mice. 8-week old female BALB/c mice were starved for 3 days and then refed with commercial diet supplemented with or without CW-P (100 mg/kg) for 8 days. Regardless of the diet used during refeeding, animal body weights and serum protein concentrations did not differ between groups. Oral treatment with CW-P normalized haemoglobin levels, liver arginase and gut mucosal weight. CW-P increased total liver proteins and also DNA and protein contents in gut mucosa. *Pleurotus* extract provided benefits in terms of macrophages activation as well as in haemopoiesis, as judged by the recovery of bone marrow cells and leukocyte counts. Moreover, CW-P stimulated humoral immunity (T-dependent and T non-dependent antibodies responses) compared to non-supplemented mice. CW-P extract from the oyster mushroom can be used to develop specific food or nutritional supplement formulations with potential clinical applications in the immunotherapy.

Keywords: immunonutrition, malnutrition, nutritional support, macrophage activation, *Pleurotus* extract

Abbreviations: CW-P, *Pleurotus* aqueous extract; M-Group, animals were killed after starvation period; M-CD-Group, animals refed with commercial pelleted diet; M-CW-P Group, animals refed CW-P.

1. Introduction

Malnutrition worldwide represents a major public health problem, leading to immune dysfunctions. Protein-energy malnutrition (PEM) has been defined as the most frequent cause of acquired or secondary immunodeficiency and as an important risk factor of microbial infection in human [1]. PEM is a common complication in patients with cancer and Acquired Immunodeficiency Syndrome [2] and it is also observed in undernourished elderly people, patients affected with anorexia and chronic diseases, and in patients undergoing chemotherapy [3-5]. Current data also highlight the link between the reproductive axis and the nutritional status, especially, undernutrition in the female [6].

It has been previously reported that PEM induced by dietetic restriction and starvation produces metabolic changes resulting in reduction of body weight and depression of immunocompetence. Digestive function, mainly in liver and small intestine is also affected [7-9].

Moreover, malnutrition modifies organism's defence processes, damaging lympho-haematopoietic organs and immune response [3]. PEM affects tissues with high turnover rate and cell proliferation as the haematopoietic tissue and induces impairment of blood cell production, leading to hypoplasia and structural changes of bone marrow [10]. In addition, cellular mechanisms as phagocytosis, inflammation, T cell activation and T cell memory are damaged. Antibody titres, cytokines production and macrophage activation are also impaired [1].

Different strategies for improving quality of life of patients under acquired immunodeficiency status by mean of immunonutritional food supports and a balanced and healthy diet have been evaluated in recent years. Immunopotentiators from natural sources, such as plants and mushroom foods components, have been used for their ability to stimulate natural and adaptive defence mechanisms [11].

The healing effects of mushrooms and its role in the diet have been recognised and documented by humankind for thousand years. Mushrooms have been commonly consumed for their flavour, high-quality proteins, vitamins, fibres and medicinal properties [12]. Many studies have evidenced that consumption of mushroom, as either food or extracts, or some specific constituents, can be used to prevent or treat several ailments [13]. Those compounds and mushroom extracts have mainly shown antibacterial, antifungal, antitumor and immunomodulatory activities [14]. Actually, edible and medicinal mushrooms are considered functional foods, nutriceuticals and a new class or drugs called “mushroom pharmaceuticals” [13, 15].

In this context, several studies have confirmed that *Pleurotus* mushrooms have been valued as nutritional foods and for their medicinal properties [16]. Various extracts and bio-substances extracted from oyster mushroom have been used against chronic diseases such as diabetes and cancer, to reduce the oxidative stress and for enhancing the immune function [16-18].

The beneficial effects of mushrooms in cancer-related malnutrition were previously reported in clinical studies [19]. Nevertheless, there is a relative lack of information regarding to the use of mushrooms derived formulations to modulate the metabolic and immunological dysfunctions accompanying malnutrition.

In present study, the effects of oral administration of an aqueous extract (CW-P) from *Pleurotus ostreatus* on the recovery of biochemical and immunological functions of malnourished BALB/c mice were evaluated. The results obtained in this research could be useful to design new immunonutritional food formulae for the prevention and treatment of several immunological disorders associated with malnutrition and for the management towards achieving a healthy state in some nutritional related disorders in women.

2. Materials and methods

2.1. Mushroom materials and preparation of crude extract

Pleurotus ostreatus strain CCEBI-3024 (Pleurotaceae) is deposited in the Culture Collection of the Centre of Studies for Industrial Biotechnology. For its maintenance, *Pleurotus ostreatus* was incubated at 37 ºC for 7 days on slants with solid dextrose-potato-agar. The botanical identification and authentication was done by Eastern Centre of Ecosystems and Biodiversity (Santiago de Cuba, Cuba).

Pleurotus ostreatus cultivation was performed by solid-state fermentation of mushroom spawn on pasteurised coffee pulp used as substrate in plastic bags of 2 kg (30x40cm) [20]. The fruiting bodies (500 g) were harvested, sliced into small pieces and were extracted with cold water between 15-20 ºC (3 mL x g of mushroom) on stirring (150 rpm) for 3 h. The suspension was filtered through sterile gauze and centrifuged at 3000 rpm for 10 minutes (Heal Force, model Neofuge 15). The resulting crude extract at a yield of 14.4 g/L culture (1.44% dried weight) was filtered with 0.2 µm bacteriological filter and kept frozen at -20 °C until use.

2.2. Mycochemical profile of CW-P

The mycochemicals contained in CW-P were estimated qualitatively according to Harbourne (1984) [21]. Dragendorff's and Wagner reagents were used for alkaloid detection. The flavonoids were tested according to the Rosemheim and concentrated sulphuric acid methods. Lieberman-Burchard and Solkowski assays were used for terpenoids identification. The presence of phenols and tannins was assessed by the reaction with $FeCl_3$. Amino acids detection was performed by the ninhydrine assay. Moreover, total phenols were quantified by the Folin-Ciocalteu reagent [22]. Carbohydrates and proteins were determined by the phenol-sulphuric method [23] and the Folin phenol reagent [24],

respectively; and the nucleotides were quantified by the Rut method [25]. All reagents for the mycochemical tests were freshly prepared following standard procedures.

2.3. Animals and treatments

Female BALB/c mice (8-week old) were purchased from the National Centre for the Laboratory Animal Production (Havana, Cuba) and housed individually. A total of 40 mice were starved for 3 days with free access to salted water. After this time, blood was collected from the orbital plexus of 10 mice and then the animals were killed (M-Group). The other animals were refed *ad libitum* for 8 days with commercial pelleted diet (M-CD-Group) supplemented with or without *Pleurotus* aqueous extract (CW-P) orally administered at a dose of 100 mg/kg of body weight per day (M-CW-P). A control group (Control) was fed with the commercial pelleted diet (ALYco®, Havana, Cuba) throughout the study. In the experiment concerning the humoral immunity, the refeeding period was extended to 14 days. Females mice were used because of their higher ability to resist the loss of energy stores during periods of starvation [26]. In a previous study, the starved BALB/c mice was suggested as an experimental model of malnutrition, and their metabolic and the immune system parameters were characterised under starvation/refeeding [27]. All experiments were approved by the Institutional Ethical Committees (University of Oriente and Centre of Toxicology and Biomedicine) and have been performed in accordance with Cuban legislation and the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.4. Biochemical analysis

2.4.1. Serum

Serum was prepared from collected blood samples and stored at -20 °C until required. Total serum proteins were measured by the Biuret colorimetric assay [28], using bovine serum albumin as a standard.

2.4.2. Liver

Liver samples were homogenised in ice-cold 0.01 mol/L phosphate buffer saline pH 7.4 (1:3 w/v). Total protein content was determined by Lowry´s method (Lowry *et al*., 1951).

The arginase activity was determined through the colorimetric reaction with α-naphthol and bromine. The enzyme-specific activity was expressed as micromoles of transformed substrate in 1 min/mg of protein [29].

2.4.3. Gut mucosa

After the small intestine was collected, the jejunum segment was rinsed thoroughly with ice-cold saline solution, opened, and blotted dry. The mucosa was scraped with a glass slide and weighed separately. Jejunal mucosa was homogenised with ice-cold phosphate buffer saline pH 6.0 (1:3 w/v). Total protein and DNA content were quantified respectively by the methods of Lowry *et al* (1951) [24] and Burton (1956) [30].

2.5. Haematological methods

Blood was collected from the retro-orbital plexus of each mouse. The blood specimens were then analysed for total and differential white blood cell counts. For differential counts, a blood sample was placed onto glass slides, fixed with methanol, and then stained was Giemsa solution. Bone marrow cells were obtained by flushing (using a syringe fitted with a 25-G needle) each isolated femur with Hank´s solution. Bone marrow cells were counted with an improved Neubauer counting chamber (Boeco, Hamburg, Germany).

Haemoglobin levels were determined by commercial Hemotest Kit (EPB Carlos J. Finlay, Havana, Cuba) based on the Drabkin reaction [31].

2.6. Innate immune response

2.6.1. Splenic cellularity

Cell suspension was obtained by homogenizing the spleen with iced Hanks´ solution. Cells were counted in a Neubauer chamber under optical microscope.

2.6.2. Peritoneal macrophage counts

Peritoneal macrophages were collected from the peritoneal cavity of mice by washing with Hank´s solution. The number was counted with Neubauer counting chamber (Boeco, Hamburg, Germany).

2.6.3. Carbon clearance from peripheral blood

The functional activity of the monocyte-macrophage system of each host was evaluated using a carbon clearance test [32]. Mice (as above, 10/group) were injected via the tail vein with 0.2 mL of a colloidal carbon suspension, consisting of 3 mL of Pelykan drawing ink 17 Black (Pelykan AG, Offenbach, Hesse, Germany), 4 mL saline, and 4 mL of 3% gelatine solution [w/v]. A 50 µL aliquot of the blood was drawn from the retro-orbital plexus into a haematocrit tube 5 min after injection of the suspension, and then immediately mixed with 4 mL of 0.1% Na_2CO_3. The concentration of colloidal carbon present was estimated by measuring absorbance at 675 nm in a Spectroquant® Pharo 300 spectrophotometer (Merck, Darmstadt, Germany). The clearance rate of carbon was expressed as the ratio of the absorbance for samples from CW-P treated (or saline-control) undernourished mice with respect to values from immunocompetent mice injected with the carbon particles (i.e., received no test substances).

2.7. Adaptive immunity: *in vivo* antibody response

Humoral immune response was evaluated through two immunization protocols with sheep red blood cells and lipopolysaccharide. Lipopolysaccharide was extracted from *Pseudomonas aeruginosa* with hot phenol [33]. Three groups (five mice each one), were designed: M-CD-Group, CW-P-Group and Control as described previously. After the starvation (day 0), mice were injected intraperitoneally (i.p) with 0.2 mL of a 25% sheep red blood cells saline solution or 50 µg of lipopolysaccharide. Seven days from the first injection, blood samples of 50 µL were drawn from the orbital plexus to measure antibody

titres by direct (sheep red blood cells) or passive (lipopolysaccharide) haemagglutination reaction, respectively. The reciprocal serum dilution which just gave agglutination was considered to be the titre. At this time, mice received the second immunization, and on day 14, antibody titres were determined.

2.8. Statistical analysis

The results were expressed as the arithmetic means ± standard deviation (SD). The Kruskal-Wallis rank test followed by the Student-Newman-Keuls test was applied to determine the significance of differences between treatments. The significance between the two groups in the experiments of carbon clearance assay was analysed by the Student's *t*-test. Differences at $p<0.05$ were accepted as significant. The software Statgraphics Plus version 5.1 (Statistical Graphics Corporation, 1994-2001) was used in all the analysis.

3. Results and discussion

Immunocompetence and nutritional deficiencies are the most important causes of mortality and morbidity in the world [1]. New strategies such as immunonutritional support are focused on the therapeutic potential of certain foods and natural products with respect to the modulation of the immune response. In this sense, the animal model of starvation used in this work has been useful in evaluating the effectiveness of nutritional and immunopharmacological interventions of natural bio-products (e.g. microalgae protein hydrolysates) [34]. The aim of the study was to investigate the effects of oral administration of an aqueous extract from *Pleurotus ostreatus* on the recovery of some biochemical and immunological functions of undernourished female mice.

3.1. Mycochemical screening and biochemical composition of CW-P.

Edible and medicinal mushrooms are commonly considered as a functional food, since the enormous variety of bioactive compounds and nutrients they contain. Fruiting bodies

and mycelia of *Pleurotus* species are largely known to possess substances that exert medicinal effects on the host´s immune system [15]. In our work, the mycochemical screening of CW-P reflected a high protein and polysaccharide contents. The main biochemical parameters in the composition of aqueous extract (CW-P), used for quality control [35], revealed 30% of protein, 36% of total carbohydrates and 0.15% of nucleotides. On the other hand, different secondary metabolites such as alkaloids, saponins, and terpenes were also detected. Qualitative evidences of quinones, amino acids, and phenolic compounds (i.e. flavonoids and tannins) were found (Table 1); total phenols represented 1.62% of CW-P.

The polysaccharides found in mushrooms are main compounds with immunopharmacological properties and can activate several immune cells [14]. Lectins, fungal immunomodulatory proteins, ribosome inactivating proteins and nucleotides derived from *Pleurotus* mushrooms have showed antiproliferative, antitumoral and immunoenhancing effects [36, 37]. On the other hand, secondary metabolites like terpenoids and flavonoids have shown antitumoral, immunoenhancing and antioxidant properties [36]. Accordingly, a cold crude extract may be a good alternative for prevention/treatment of diseases related with oxidative stress and immunodepression.

3.2. Effects of malnutrition and refeeding on body weight and biochemical parameters in serum and digestive system of BALB/c mice.

It is well-documented that malnutrition due to starvation, disease or ageing produces a series of metabolic alterations that often result in deterioration of digestive system activity and compromised the immune function [1, 3].

Anthropometric measures have been used as criteria of nutritional status assessment in nutritional practice [38]. After 72 h of starvation significant differences in body weight were found. Mice lost about the 25% of the initial body weight from 18.5±0.3 g up to

13.3±0.2 g. M-CD and M-CW-P groups progressively recovered the body weight during 8 days of refeeding (18.7±1.5; 19.2±1.6 g, respectively) compared with control group (19.1±0.8 g) ($p<0.05$). Although the weight was gradually recovered during refeeding, CW-P did not increase the body weight in mice. As there are no previous reports about the consumption of mushroom products by malnourished mice, some aspects should be considered for further evaluations of this parameter, such as the biomodel used, the extension of the refeeding period and the composition of diet.

No significant differences on average daily and food consumptions were found between the refed groups. The energy and protein intakes for each animal were similar in both re-nutrition treatments (data not shown).

Pathological variations in serum proteins are common in PEM and they are often related to hepatic failure. No significant differences in serum protein levels and liver to body weight ratio were observed in animals orally supplemented with the *Pleurotus* extract compared with the control and M-CD groups ($p<0.05$) (Table 2). However, statistically significant differences were observed in liver protein-content. The administration of CW-P during the re-nutrition produced a marked recovery of mice liver protein-content compared with M and M-CD groups ($p<0.05$) (Table 2). We concluded that CW-P restored the normal function of protein biosynthesis in liver after the refeeding period although the changes in serum protein content might be reflected slowly. More sensitive markers like albumin and transferrin have been considered as good indicators in malnutrition studies because their concentrations decrease rapidly in acute diseases and they can recover in a relatively short time [38].

Arginase which catalyses the catabolism of arginine to urea and ornithine is highly expressed in liver. Previous studies described that the arginase activity could increases approximately ten-folds during liver injury and appears as a good biochemical marker to

evaluate the hepatic function, even compared with alanine aminotransferase and aspartate aminotransferase activities [39, 40]. A significant statistically decrease in arginase activity was observed in animals fed with CW-P (22.69±1.5 μmol/min /mg of protein) compared to the M-CD group (40.22±0.14 μmol/min /mg of protein). The enzymatic activity in the control group was 18.25±0.87 μmol/min /mg of protein ($p<0.05$). The decrease of arginase activity in malnourished mice after administration with CW-P reflected the restoration of liver functional capacity involved in protein anabolism and the reduction of urea synthesis. Further studies, in which the changes of other biomarkers such as alanine aminotransferase and aspartate aminotransferase, can be detected at the same time that arginase are needed for a better understanding of liver biosynthetic and detoxifying functions.

The gastrointestinal tract has a high impact on human health through the host metabolism, physiology, nutrition and immune functions [41]. In PEM there are reductions in nutrient absorption and depression of the immune system in gut associated lymphoid tissues [1].

The functionality and integrity of small intestine was also deteriorated. Mucosal weight, mucosal DNA and protein contents were lower in animals fed with the standard diet. Administration of CW-P during refeeding period provided beneficial effects and improved functional alterations ($p<0.05$) (Fig.1).

Nutritional support with CW-P produced beneficial effects in the intestinal tract. The increase of mucosal weight in association with high values of protein and DNA content suggests a higher rate of protein biosynthesis and could be related with the recovery of gastrointestinal tract function. Some components present in *Pleurotus ostreatus* mushroom could re-establish intestinal architecture after oral consumption. In general, the enteral nutrition is considered the first method of feeding in critical patients as hospitalized children [38], and thus mushrooms substances might be potential candidates for using in immunonutritional diets. Some studies with mushrooms reported the biological potential of

polysaccharides at intestinal level by means of the stimulation of gut associated lymphoid tissues and intestinal macrophages [42]. Therefore, it may contribute to the integrity of the gastrointestinal epithelium and mucosa restoration.

3.3. Influence of malnutrition and refeeding on haematological parameters and monocyte-macrophage system

PEM triggers atrophy in bone marrow and reduces the number of leukocytes [3]. CW-P stimulated the haemopoiesis as judged by an increase in bone marrow cells, haemoglobin levels and leukocyte counts. Haematopoiesis measured as bone marrow cells and leukocyte counts in peripheral blood was enhanced in animals administered additionally with CW-P extract, and this effect was statistically higher than the M-CD group ($p<0.01$) (Fig.2).

Moreover, haemoglobin content significantly decreased in malnourished mice at day 3 with respect to the control group. Eight days after supplementation with CW-P, treated animals showed a significant recovery of haemoglobin content compared to animals fed with standard diet ($p<0.05$) (Table 2). The result indicated that CW-P could stimulate differentiation of bone marrow cells and lymphoid tissues by haematopoietic cytokines like colony-stimulating factor, thus providing a promptly recovery.

The monocyte-macrophage system plays a pivotal role in a broad range of innate and specific immune responses. In general, the phagocytic function is severely deteriorated during the malnutrition status [1]. The number of peritoneal macrophages significantly decreased after malnutrition period (M group). Oral administration of the CW-P extract for 8 days increased the number of resident macrophages in peritoneal cavity compared with the M-DC group ($p<0.05$) (Fig. 3).

In addition, the effect of *Pleurotus ostreatus* mushroom extract on phagocytic activity of Kupffer cells and splenic macrophages in mice was evaluated by the carbon clearance test. The oral administration of CW-P at a dose of 100 mg/kg stimulated the phagocytic activity

in comparison with the standard diet group. The rate of carbon clearance at 5 min was shorter than in the M-CD group (1.4±0.1 *vs* 1.9±0.1) ($p<0.05$).

Our findings suggest that CW-P can modulate the biological activity of the monocyte-macrophage system. Nevertheless, CW-P does not increase spleen weight and splenic cell counts ($p<0.05$) (Table 3). The augmentation of phagocytic activity may be owing to the activation of phagocytes and not by an increase in the number of total phagocytes.

The effects on intestinal tract linked to macrophage activation might be influenced by a partial absorption of bio-compounds from *Pleurotus ostreatus*, or by the stimulation of the gut associated lymphoid tissues. The intestinal macrophages are major cells in the human monocyte-macrophage system and are preferentially localized in the subepithelial region [41]. However, further studies are necessary to clarify how CW-P activates the macrophages after its oral administration.

The macrophage activation could be specifically related to the presence of active myco-chemicals such as mushrooms polysaccharides. Traditionally, macrophages and dendritic cells are considered the main target cells of mushroom carbohydrates. Some receptors that recognise polysaccharides domains of mushrooms have been identified, but Dectin-1 is the most known and play a crucial role in coordinating immune defence against microbes and tumour cells destruction [43]. Nevertheless, mechanism of action of several orally administered bio-substances from mushrooms is still unclear. Among various myco-chemicals, it has been suggested that only fragments of polysaccharides partially hydrolysed or degraded after ingestion might bind to gut epithelia and exert localised and/or systemic effects on the immune system or the mechanisms could be mediated *via* a nonspecific intestinal absorption [13]. Although most of the bio-components in CW-P extract could be probably implicated as immunomodulatory agents, more evidences are required to link the observed actions to any of the identified bio-components.

3.4. Effects of malnutrition and refeeding on antibody immune response

Although cell-mediated immunity is severely affected in PEM, the atrophy of lymphoid tissues leads to a decrease of circulating and the splenic B cell numbers [9]. However, the role of humoral immune response in malnourished mice is not well documented.

After seven days of immunization, there were no significant differences in haemagglutination titres to sheep red blood cells and lipopolysaccharide between the M-CD and M-CW-P groups (data not shown). However, antibody production by B cells after 14 days of immunization with sheep red blood cells or lipopolysaccharide was significantly higher compared with mice refed with commercial diet ($p<0.05$) (Fig. 4). Anti-sheep red blood cells antibodies (directed against a T-dependent antigen) titres might also suggest the stimulation of cellular immunity.

Other studies indicated that the humoral response might respond to malnutrition depending on malnutrition type (e.g. acute *vs.* chronic, protein malnutrition *vs.* energy restriction) [44].

The term immunonutrition was introduced as emergent subject in last years [45]. The concept serves not only for supporting energy and nutritional substrates but also for other essential substances that could enhance immune response in undernourished patients. Recently, the pharmaconutrition concept involve nutrients studied as therapeutic components administered in pharmacological doses and focused on specialized nutritional support to a study active therapeutics on immune function [46]. However, there are no specific diets designed for different physiological conditions. Future efforts should therefore define the most effective nutrients/substances and immunoenhancing diets for their use in different patient groups.

In addition, the occurrence of immunodeficiencies associated to malnutrition in female could be linked to many factors, such as anaemia by iron loss, the prevalence of anorexia

nervosa, pregnancy, lactating women, among others [6, 47, 48]. The use of bioactive preparations from mushrooms could be an attractive option to introduce new prophylactic strategies to prevent and reduce the negative effects caused by malnutrition in women, thus increasing their quality of life.

4. Conclusions

The current work evidenced that the CW-P extract from the oyster mushroom contain mycosubstances that can restore the homeostasis and the host defence mechanisms *in vivo* by potentiating biochemical functions as well as innate and specific immune responses in malnourished mice. Our study also supports the hypothesis that some biocompounds and bionutrients might be absorbed through intestinal tract and exert systemic effect on immune function. Thus, CW-P could be used to develop specific enteral formulations with potential applications in the immunotherapy and as immunonutritional support during recovery of the metabolic and immunological disorders associated with malnutrition. This study is a contribution to the knowledge of the immunonutritional properties of *Pleurotus* mushroom and suggests its prospective use in immunocompromised people with special nutritional requirements (e.g. female with risk of malnutrition due to metabolic and/or pathologic conditions).

Conflict of interest

The authors declare no conflict of interests.

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Table 1. Mycochemicals constituents of cold crude extract from *Pleurotus ostreatus* fruiting bodies.

Metabolites	**Assays**	**Evidence**
Alkaloids	Dragendorff	+++
	Wagner	+
Saponins	Foam formation	+
Flavonoids	Concentrated H_2SO_4	+
Terpenoids	Solkowski	+
	Lieberman-Burchard	+
Quinones	Borntrager	+
Amino acid	Ninhidrine	++
Tannins and Phenolic compounds	$FeCl_3$	+

Note: The mycochemicals contained in CW-P were estimated qualitatively according to Harbourne (1984). Three replicates were used for each assay. Legend: (+) present, (++) mild, (+++) marked.

Table 2. Effect of CW-P oral administration on biochemical parameters in serum and digestive system of BALB/c mice.

	Control	M	M-CD	M-CW-P
Haemoglobin (g/dL)	13.47 ± 0.88^{ab}	8.57 ± 0.92^{c}	12.18 ± 0.7^{b}	14.18 ± 1.17^{a}
Serum total protein (g/dL)	4.63 ± 0.87^{a}	1.93 ± 0.32^{b}	3.6 ± 0.2^{ab}	5.45 ± 1.5^{a}
Liver mass index (mg/100g) ns	58 ± 3.4	51 ± 5.2	52 ± 2.3	56 ± 2.5
Liver protein (mg/g)	138.3 ± 12.9^{b}	52.4 ± 12.2^{d}	102.8 ± 8^{c}	160.3 ± 30^{a}

All values are expressed as the arithmetic mean ± SD of 10 mice. (ns) Non significant differences. Different letters in the same row indicate statistically significant differences according to the Kruskal–Wallis rank test followed by the Student–Newman–Keuls test ($p<0.05$). Subscript legend: (a) ≠ (b) ≠ (c) ≠ (d) and (ab) means non differences from (a) and (b).

Table 3. Effect of CW-P oral administration on cellularity and spleen mass index of BALB/c mice.

	Control	**M**	**M-CD**	**M-CW-P**
Spleen Mass Index (mg/100g)	380 ± 0.1^{a}	270 ± 0.1^{b}	430 ± 0.1^{a}	430 ± 0.01^{a}
Spleen cellularity (x10^{7}/spleen)	9.5 ± 0.1^{a}	3.0 ± 0.1^{b}	10.6 ± 0.2^{a}	10.4 ± 0.1^{a}

Cell suspension was performed by homogenizing the spleen with iced Hanks´ solution. Cells were counted in a Neubauer chamber under optical microscope (10^{7}cells / spleen). All values are expressed as the arithmetic mean ± SD of 10 mice. Means without the same letter are significantly different according to the Kruskal–Wallis rank test followed by the Student–Newman–Keuls test ($p<0.05$). Subscript legend: (a) ≠ (b)

Figure captions

Fig. 1. Effect of CW-P oral administration on mucosal weight (A), protein mucosal (B) and DNA contents (C) in jejunum of BALB/c mice.

Results are expressed per ten centimetres of intestine. All values are expressed as the arithmetic mean ± SD of 10 mice. Different letters indicate significant differences among groups (Kruskal Wallis, Student Neuman Keuls, $p<0.05$). Subscript legend: (a) ≠ (b) ≠ (c)

Fig. 2. Effect of CW-P oral administration on haemopoiesis of BALB/c mice.

All values are expressed as the arithmetic mean ± SD of 10 mice. Different letters indicate significant differences among groups (Kruskal Wallis, Student Neuman Keuls, $p<0.01$). Subscript legend: (a) ≠ (b) ≠ (c) ≠ (d) and (ab) means non differences from (a) and (b).

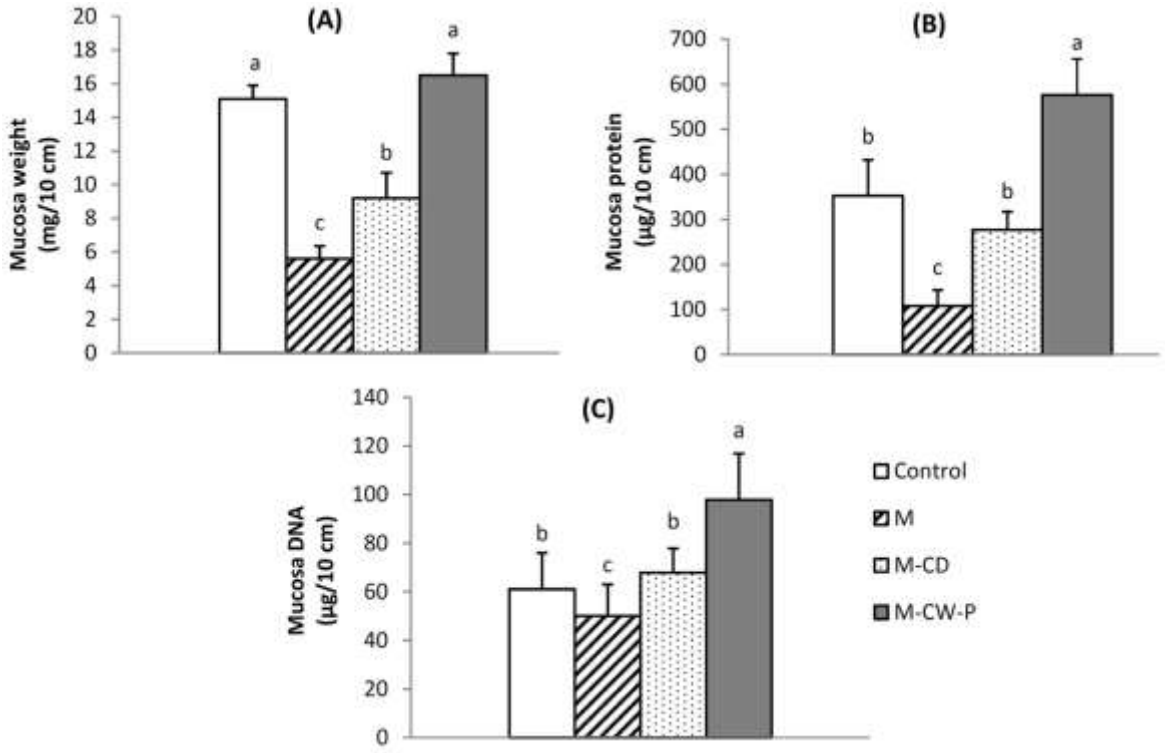
Fig. 3. Effect of CW-P oral administration on peritoneal macrophages BALB/c mice.

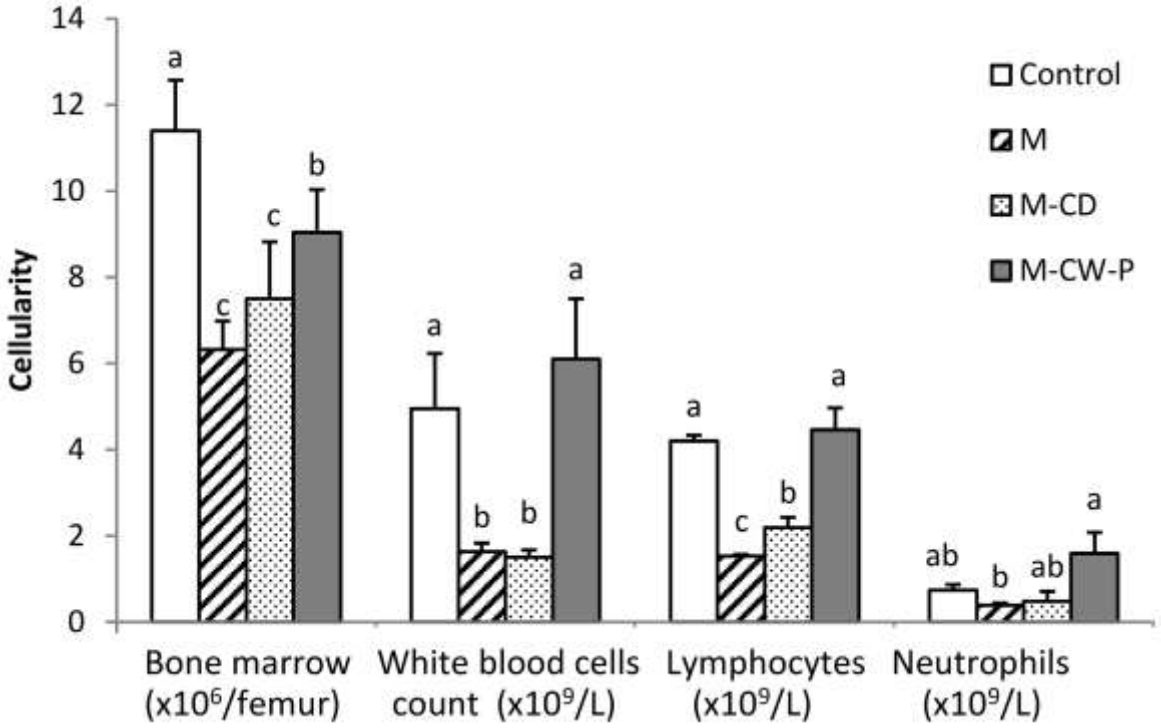
Macrophages were isolated from the peritoneal cavity by lavage with Hanks´ solution and counted with a Neubauer chamber. All values are expressed as the arithmetic mean ± SD of 10 mice. Different letters indicate significant differences among groups (Kruskal Wallis, Student Neuman Keuls, $p<0.05$). Subscript legend: (a) ≠ (b) ≠ (c)

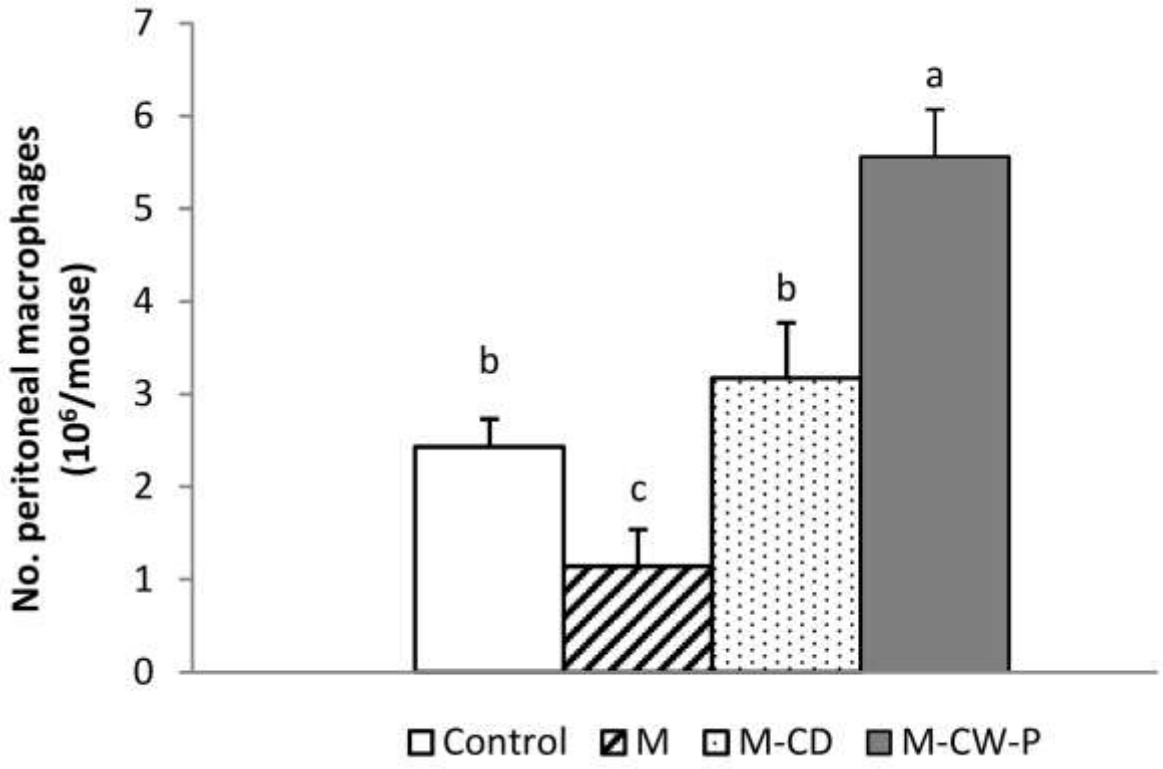
Fig. 4. Effect of CW-P oral administration on humoral immune response of BALB/c mice, against: (A) T-dependent (sheep red blood cells) and (B) T-independent (lipopolysaccharide) antigens at 14 days of inoculation.

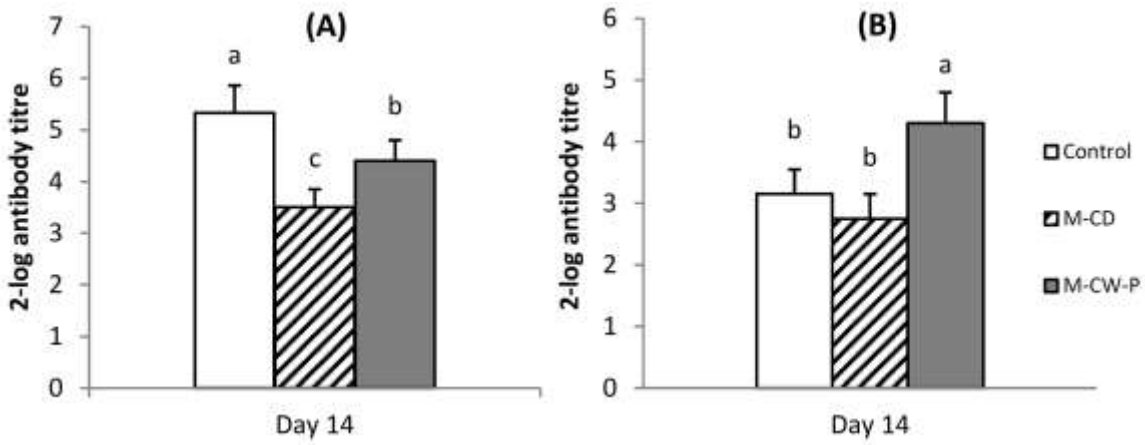
After the starvation period (day 0) and after seven days, five animals per group were injected i.p. with 0.2 mL of a 25% of SRBC saline solution and 0.2 mL of LPS (50µg/mouse). At 14 days from the first injection the anti-SRBC and anti-LPS antibodies titres were measured by active and passive haemagglutination, respectively. The reciprocal serum dilution which just gave agglutination was considered to be the titre. All values are expressed as the arithmetic mean ± SD of 5 mice. Different letters indicate significant

differences among groups (Kruskal Wallis, Student Neuman Keuls, $p<0.05$). Subscript legend: (a) ≠ (b) ≠ (c)


(A)
Mucosa weight (mg/10 cm)
20
18
16
14
12
10
8
6
4
2
0
a
c
b
a
(B)
Mucosa protein (μg/10 cm)
700
600
500
400
300
200
100
0
b
c
b
a
(C)
Mucosa DNA (μg/10 cm)
140
120
100
80
60
40
20
0
b
c
b
a
Control
M
M-CD
M-CW-P


Cellularity
14
12
10
8
6
4
2
0
a
c
b
a
b
b
a
a
c
b
a
ab
b
ab
a
Control
M
M-CD
M-CW-P
Bone marrow (x10⁶/femur)
White blood cells count (x10⁹/L)
Lymphocytes (x10⁹/L)
Neutrophils (x10⁹/L)


No. peritoneal macrophages
(10⁶/mouse)
7
6
5
4
3
2
1
0
a
b
b
c
Control
M
M-CD
M-CW-P


(A)
2-log antibody titre
7
6
5
4
3
2
1
0
a
c
b
Day 14
(B)
2-log antibody titre
6
5
4
3
2
1
0
b
b
a
Day 14
Control
M-CD
M-CW-P