Food Chemistry 134 (2012) 758–765

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vitro and *in vivo* protective effects of fermented preparations of dietary herbs against lipopolysaccharide insult

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ARTICLE INFO

Article history:
Received 17 August 2011
Received in revised form 27 December 2011
Accepted 28 February 2012
Available online 6 March 2012

Keywords:
Lipopolysaccharide
Fermented herbs
RAW264.7 cells
Probiotics
Gut permeability

ABSTRACT

Lipopolysaccharide (LPS) is known to produce endotoxic shock by triggering systemic inflammatory responses. Here, we evaluated the protective effects of three fermented/re-fermented herbs, Rhizoma Atractylodis Macrocephalae, Massa Medicata Fermentata, and Dolichoris Semen, in an LPS-mediated inflammatory insult, either individually *in vitro* using RAW264.7 cells or in combination in *in vivo* using rats. In general, each of the fermented herbs showed appreciable *in vitro* anti-inflammatory activity, although the degree of this activity varied with the herb used. Moreover, a mixture of fermented herbal extracts in combination with probiotics significantly attenuated the blood endotoxin and CRP levels, as well as the gut permeability, and significantly augmented the intestinal *Lactobacillus* spp. colonisation in LPS-treated rats. However, these effects were not observed following the administration of the corresponding mixture of unfermented herbal extracts. Thus, our results highlight the beneficial impacts of the use of fermented herb products with probiotics to combat LPS-mediated inflammatory insults.

1. Introduction

LPS, or endotoxin, is an integral component of gram-negative bacteria and is one of the major causative agents of systemic inflammatory responses that lead to a number of adverse effects, including: endotoxic shock, tissue injury, sepsis, multiple organ failure, and death. Activated macrophages are known to be the dominant proinflammatory cells involved in the onset of the systemic inflammatory responses. More specifically, these cells are responsible for most of the cellular and molecular pathophysiology of sepsis due to their production of a number of cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, IL-8, and IL-12 and other proinflammatory molecules, including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide (NO). Endotoxin has been shown to destabilize gut permeability, permit bacterial translocation, and alter the host immune defence mechanisms, which, as a result, collectively enhance the risk of developing infections from the constituents of the enteric microbiota (Deitch, Berg, & Specian, 1987; Deitch et al., 1989).

Substantial evidence has indicated the anti-inflammatory effects of dietary herbs, which manifest through a number of mechanisms (Kaplan et al., 2007). Accordingly, Rhizoma Atractylodis Macrocephalae (RAM), Massa Medicata Fermentata (MMF), and Dolichoris Semen (DS, also known as hyacinth bean), which are utilised in various dietary preparations in China, Korea, Japan, and India, are frequently used either alone or in mixed herbal formulations for the treatment of a number of inflammatory disorders (Fan et al., 2005; Kim et al., 2007; Ryu et al., 2011).

Fermentation is frequently used to break down or convert certain undesirable substrate components into compatible ones and this process can also increase the activities of the biological substrates by modifying naturally occurring molecules such as isoflavones, saponins, phytosterols, and phenols. The beneficial health effects and the anti-inflammatory activities of fermented herbal or food products, either alone or in combination, are well documented (Deiana et al., 2002; Parvez, Malik, Ah Kang, & Kim, 2006; Telekes et al., 2007). Additionally, the probiotics used for fermentation may also exert health-promoting effects (Parvez et al., 2006), and the anti-inflammatory properties of probiotics have also been reported by a number of studies (Grimoud et al., 2010; Parvez et al., 2006).

These findings prompted us to evaluate the protective effects of the fermented/re-fermented extracts of RAM, MMF, and DS on an LPS-driven inflammatory-insult. Accordingly, we examined the anti-inflammatory effects of the fermented/re-fermented herb products (along with the associated probiotics) individually on

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doi:10.1016/j.foodchem.2012.02.175

LPS-treated RAW264.7 cells *in vitro*, and in combination (fermented herbal formulation, FHF) in rats exposed to LPS *in vivo*, where a commercial colostrum preparation was used as the standard biological-in-origin anti-inflammatory agent. The re-fermentation of MMF was performed using *Leuconostoc mesenteroides*, whereas *Bacillus licheniformis* was employed for the fermentation of both RAM and DS.

Leuconostoc spp. are Gram-positive, non-motile and asporogenous bacteria (Hemme & Foucaud-Scheunemann, 2004). They are facultative anaerobic cocci, usually occurring in pairs or short chains (Hemme & Foucaud-Scheunemann, 2004), although the cell morphology can vary with the growth conditions. On the other-hand, *Bacillus* spp. are rod-shaped, sporulating, obligate or facultative anaerobic bacteria which test positive for the enzyme catalase (Turnbull, 1996). The selection of the above two bacterial spp. for the herbal-specific fermentation in our study was based on the research outcome of a local herbal pharmaceutical company (Korea Medicine Biofermentation Co., Ltd., Seoul, Korea) in collaboration with us, which revealed that the herbal fermentation/re-fermentation was optimum when mediated by *L. mesenteroides* (for MMF) or by *B. licheniformis* (for RAM and DS). This is also in agreement with earlier studies which have shown that *Leuconostoc* spp. play an important role in the fermentation of a wide range of products including foods, such as dairy products, meat, fish, cereals, vegetables, and fruits as well as serve as probiotics (Hemme & Foucaud-Scheunemann, 2004), suggesting that these bacteria are of worthy choices as a starter to mediate the re-fermentation of MMF, which is composed of a mixture of several fermented herbs and grains. On the other hand, *B. licheniformis*, which is listed in the third edition of the Food Chemicals Codex (1981) as a source of carbohydrase and protease enzymes, has been safely used for large-scale industrial fermentation as well as in commercial human and animal probiotic products (de Boer, Priest, & Diderichsen, 1994; Hong, Duc, & Cutting, 2005). Substantial evidence has revealed that *Bacillus* spp. play an important role in the fermentation of different legume products like beans (Allagheny, Obanu, Campbell-Platt, & Owens, 1996), as well as herbs (Hsu & Chiang, 2009; Li, Shen, Liu, & Zhang, 2006; Li, Zhang, & Shen, 2006), suggesting that these bacteria are of suitable options as starter for the fermentation of RAM and DS.

Finally, we also studied the anti-inflammatory activity of a combination of laboratory-unfermented herbs (unfermented herbal formulation, UHF) in LPS-treated rats to compare the *in vivo* effectiveness of this combination to that of corresponding FHF.

2. Materials and methods

2.1. Herbal extraction and fermentation/re-fermentation

Dried herbs were purchased from the Department of Medicine, Dongguk International Hospital (Goyang, Korea). The extraction and the fermentation/re-fermentation of the herbs were performed according to our laboratory-optimised procedure. Briefly, 20 g of dried powder from each herb was mixed with 200 ml of boiled Milli-Q water, and this mixture was subjected to ultrasonication at 70 °C to disperse the particles and then incubated at 70 °C for 3 h in a water bath under continuous shaking. Following this procedure, the samples dedicated for fermentation/re-fermentation were either supplemented with glucose (2% w/v, for RAM and MMF) or Luria–Bertani broth powder (2.5% w/v, for DS). The resultant products were then autoclaved for 20 min at 121 °C, which in addition to sterilization of the samples and killing the already existing microbes that were involved in the natural fermentation of MMF also served to decoct the samples. After cooling the preparations to room temperature, the samples dedicated for fermentation/re-fermentation were inoculated with a fresh subculture (2% v/v) of bacteria (*L. mesenteroides* for MMF, and *B. licheniformis* for both RAM and DS) and were fermented for 24 h either at 35.4 °C (*L. mesenteroides*) or 31 °C (*B. licheniformis*). The corresponding unfermented samples were prepared in a similar way, except that they received 2% (v/v) of the respective sterile bacterial culture medium instead of the bacterial inoculum. For the *in vivo* studies, the corresponding laboratory-unfermented or fermented/re-fermented herbal extracts were combined in equal volumes and mixed vigorously to produce the UHF and FHF, respectively. Finally, each of the preparations was subjected to low speed centrifugation to sediment the particles, and the supernatant phase of the resultant products was used for the experiment.

2.2. Cell culture

The RAW264.7 murine macrophage cell line was suspended in DMEM supplemented with 10% heat inactivated faetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in this medium at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

2.3. Cytotoxicity assessment

The cell viability was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) as the chromophore. After three to four cycles of sub-culturing, the RAW264.7 cells were seeded into 24-well plates at a density of 2×10^5 cells/well and were incubated overnight. Subsequently, the cells were treated for 24 h with an individual fermented herb at doses that corresponded to 5, 10, 25, 50, and 100 μl of the extracted herbal preparation per ml of culture medium. The control cells were treated with sterile water instead of an herbal extract. MTT was added to the cells 3 h prior to the end of the treatment schedule, at a final concentration of 0.5 mg/ml. After the completion of the MTT reaction, the culture media were removed carefully from the wells, and DMSO was added to the cells in order to liberate and dissolve the formazan crystal products. Following this, absorbance was read at 570 nm using a microplate reader (Spectramax Plus, Molecular Devices, Sunnyvale, CA, USA). The viability of the control cells, in terms of their absorbance, was considered to be 100%.

2.4. Determination of the expression of key inflammatory genes in RAW 264.7 cells by quantitative real-time PCR (qRT-PCR)

After three to four to 4 cycles of sub-culturing, RAW264.7 cells were seeded at a density of 8×10^5 cells/well in 6-well plates. Following an overnight culture, the cells were treated for 24 h with an individual fermented herb at doses that corresponded to 50 and 100 μl of the extracted herbal preparation per ml of culture medium. The control cells and cells that were assigned to get treatment with LPS alone received sterile water instead of the herbal extract. Following this treatment, LPS (from *Pseudomonas aeruginosa*, Sigma–Aldrich, St. Louis, MO, USA), which was diluted in sterile PBS at pH 7.4, was added to each non-control well (control received PBS only) at a final concentration of 10 μg/ml. The control cells received PBS only. The cells were incubated under these conditions for an additional 24 h before they were used in the gene expression experiments.

The total cellular RNA was extracted using a commercial Trizol® reagent kit (Invitrogen, Carsbad, CA, USA) according to the kit manufacturer's instructions. The purity and concentration of the extracted RNA was determined by spectrophotometry. An equal amount of the RNA preparation (2 μg) was reverse transcribed for the generation of cDNA using a Sprint™ RT Complete Oligo-$(dT)_{18}$ cDNA synthesis kit (Clontech, Mountain View, CA, USA) according