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Viability and dose–response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice

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Previous studies have indicated that the lactic acid bacterium *Lactobacillus rhamnosus* HN001 can enhance immune function in mice, following oral delivery. However, the influence of bacterial cell viability on immunoenhancement, and the optimum dose of HN001 required for this effect, have not been determined. In the present study, both live and heat-killed preparations of *L. rhamnosus* HN001 were shown to enhance the phagocytic activity of blood and peritoneal leucocytes in mice, at a dose of 10^9 micro-organisms daily. In contrast, only live HN001 enhanced gut mucosal antibody responses to cholera toxin vaccine. Feeding mice with 10^7 viable HN001/d for 14 d was shown to enhance the phagocytic capacity of blood leucocytes, with incremental enhancement observed at 10^9 and 10^{11} daily doses. In contrast, a minimum dose of 10^9 viable HN001/d was required to enhance the phagocytic activity of peritoneal leucocytes, and no further increment was observed with 10^{11} daily. This study demonstrates that *L. rhamnosus* HN001 exhibits dose-dependent effects on the phagocytic defence system of mice, and suggests that while the innate cellular immune system is responsive to killed forms of food-borne bacteria, specific gut mucosal immunity may only be stimulated by live forms.

***Lactobacillus*: Immune enhancement: Viability: Dose–response: Phagocytosis: Antibody: Mucosal immunity**

Lactic acid bacteria (LAB) are major constituents of the normal intestinal microflora, and in addition, represent the predominant fermentative microbes in dairy foods such as yogurt and cheese (Salminen *et al.* 1998*a*). It is generally perceived that consumption of foods containing LAB can be beneficial to health (Macfarlane & Cummings, 1999), and different strains of LAB have been shown to affect a number of host physiological functions following oral delivery (Salminen *et al.* 1998*a*). Among these, certain well-defined strains have been shown to affect immune function (Gill, 1998), and thus recent research has sought to characterise these strains such that they might be incorporated into immune-enhancing functional foods (Salminen *et al.* 1998*b*).

In the course of such research, it is important not only to define the particular aspects of host immunity that are affected, but also to be able to optimise the immune-enhancing properties of the particular LAB strain under consideration, in order to support development of the most effective preparation for human use. Issues of particular importance include determining the effects of microbial cell viability on immunoenhancing efficacy, and defining the minimum dose of the micro-organism that is required to enhance immunity. In the former case, technical difficulties in maintaining bacterial survival following commercial production (Gardiner *et al.* 2000) raise the question as to whether a decline in cell viability could deleteriously affect the immunoenhancing efficacy; in the latter case, the economics of producing food-quality bacterial cultures dictate that production costs can be minimised in products that contain low numbers of bacterial cells. Both of these issues are therefore relevant to the commercialisation of probiotic-containing functional foods, and can be addressed (in the first instance) by studying appropriate animal models.

Our laboratory has recently identified an immunoenhancing strain of *Lactobacillus* (*L. rhamnosus* HN001), isolated originally from Cheddar cheese. In animal studies, mice which were fed HN001 for 10 or 28 d were shown to have enhanced blood and peritoneal leucocyte phagocytic capacity, and enhanced antibody responses to vaccine antigens (Gill, 1998; Gill *et al.* 2000). *L. rhamnosus* HN001

Abbreviation: LAB, lactic acid bacteria.

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exhibits good *in vitro* survivability under low pH–high bile concentrations (Prasad *et al.* 1999), and has been demonstrated *in vivo* to transiently survive in murine or human gastrointestinal tracts following dietary delivery (Tannock *et al.* 2000; PK Gopal and HS Gill, unpublished results), thus satisfying the criteria required for its designation as a probiotic (Salminen *et al.* 1998*a*).

While the majority of reports of immune enhancement by probiotic LAB have suggested that gut survival is the important factor determining physiological effects of the micro-organisms, a recent study has suggested that immune enhancement may occur independently of microbial persistence in the gastrointestinal tract (Donnet-Hughes *et al.* 1999). In addition, some animal studies have indicated that non-viable preparations of LAB can also be effective in enhancing immunity (Perdigon *et al.* 1986; Portier *et al.* 1993), raising the proposition that foods containing killed bacterial cells may also effect immune enhancement. Very few studies have directly compared the impact of viable *v.* non-viable LAB on immunity, particularly in cases where the LAB strain in question has already been shown to enhance immunity when used in viable form as a probiotic (Perdigon *et al.* 1986). The effects of cell viability on the immunoenhancing efficacy of *L. rhamnosus* HN001 are unknown.

In our previous studies in mice, oral delivery of 10^9 *L. rhamnosus* HN001/d was shown to enhance immune responses (Gill, 1998; Gill *et al.* 2000). Although 10^9 micro-organisms represents a typical daily dose for murine studies (Perdigon *et al.* 1986, 1988), research elsewhere has indicated that some strains of LAB are able to enhance immunity at lower doses (Portier *et al.* 1993). The dose response of *L. rhamnosus* HN001 in mice is unknown, and the question remains, therefore, whether the immune-enhancing effects of this strain could be retained at a lower dosage or, conversely, whether doses higher than 10^9/d would confer significant increments in immunoenhancement.

The aims of the present study were two-fold: to investigate probiotic viability and dose–response effects on immune enhancement in mice. Using *L. rhamnosus* HN001 as a benchmark LAB strain that is known to enhance immunity when used in viable form (i.e. as a probiotic), we first wanted to compare these responses to those observed in mice fed non-viable HN001 cells. The second aim of this study was to determine the optimum dose of HN001 required to enhance immune function when used as a viable probiotic.

Materials and methods

Mice

BALB/c mice, aged 8–10 weeks, were bred and maintained at the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. Animals were housed in individual cages at 22 ± 1°C under a diurnal photoperiod, and fed a standard mouse chow with *ad libitum* water. Prior to experimentation, mice were acclimatised to a milk-based diet comprising (g/kg): skimmed-milk powder (protein 380) 526, mineral mix 50, vitamin mix 50, cellulose 10, cornflour 284, corn oil (fat) 80. The acclimatisation diet was offered for 7–10 d prior to commencement of the experiments and continued throughout their duration; fresh water was offered *ad libitum*.

Bacteria

Lactobacillus rhamnosus HN001 was cultured to mid log-phase as described previously (Prasad *et al.* 1999); cells were harvested and resuspended in skimmed milk (New Zealand Dairy Research Institute, Palmerston North, New Zealand). HN001 cells were incorporated at three different concentrations into skimmed milk, such that 50 μl would yield calculated doses of 10^7, 10^9 or 10^{11} organisms. Cultures were supplied fresh for each daily feeding. In some cases, HN001 cells were killed prior to their inclusion in milk by heat-treatment (100°C/15 min). In each case, mice were fed 50 μl HN001-supplemented skimmed milk daily for 14 d by direct feeding; control mice received plain (unsupplemented) skimmed milk.

Experimental protocol

Two main experiments were conducted. The first experiment was designed to determine the effects of bacterial cell viability on immune enhancement. For this, eighteen female mice received daily doses of milk supplemented with 10^9 viable or heat-killed HN001 for 14 d, while eighteen control mice received unsupplemented milk. Mice were immunised orally on day 0 and day 7 with 25 μg cholera toxin vaccine (Sigma, St Louis, MO, USA) in 25 μl 0·1 M-NaH_2CO_3. Mice were killed as described later; blood and peritoneal cell samples were prepared, spleens were dissected and intestinal mucus washes were obtained.

The second experiment was designed to determine the dose response of immune enhancement conferred by HN001 in viable form. Groups of eighteen male mice received daily doses of milk supplemented with 10^7, 10^9 or 10^{11} viable HN001 for 14 d, while eighteen control mice received unsupplemented milk. At the end of this period, mice were killed as described later, and blood and peritoneal cell samples were prepared.

Tissue samples

Mice were killed via overdose of the inhalation anaesthetic isofluorane. Blood samples were obtained from mice by cardiac puncture and put into tubes containing EDTA. Peritoneal cell samples were obtained by lavage of the peritoneal cavity with warm RPMI 1640 tissue culture medium (Gibco Life Technologies, Auckland, New Zealand) containing 100 ml fetal calf serum/l. Spleens were dissected and weighed individually. The small intestine was recovered, and the contents flushed with 1·5 ml warm PBS; particulate material was removed by centrifugation and the remaining supernatant fluid was used to measure mucosal antibody response to cholera toxin.

Immunological assays

Cell samples and intestinal mucus washes were prepared as

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described previously (Gill *et al.* 2000). Leucocytes were identified in blood samples by morphological characteristics using a FACScalibur flow cytometer (Becton-Dickinson Instruments, Cambridge, MA, USA), and cell counts were performed automatically using a standardised FACS protocol. Phagocytosis was assessed in blood and peritoneal cell preparations by flow cytometric analysis of the uptake of fluoresceinated *Escherichia coli*; results were expressed as the proportion (percentage) of phagocytically active cells in each sample, as described previously (Gill *et al.* 2000). Antibody responses to cholera toxin antigen were assessed in intestinal mucus samples by ELISA, using an alkaline phosphatase-conjugated rat anti-mouse whole immunoglobulin secondary antibody (Silenus, Victoria, Australia) as described previously (Gill *et al.* 2000); results were expressed as specific antibody units/ml mucus sample against a 2000 U hyperimmune serum/ml standard. For assays of antibody responses, the intestinal mucosal response of each mouse was assessed individually (n 18 for control and test groups); in order to provide sufficient cells from each group to complete phagocytosis assays, samples were pooled from every second mouse within each group (n 9 for control and test groups).

Statistical analyses

Differences in immune responses were compared for each test diet and treatment against the respective control by analysis of variance, with Fisher's least-squares difference to detect significant differences between treatment levels. Where data were non-normally distributed, two sample Mann-Whitney U tests were employed instead.

Results

Viability effect of HN001 on enhancement of phagocytosis and antibody production

Mice which received either live or heat-killed HN001 at a dose of 10^9/d had significantly higher blood and peritoneal cell phagocytic responses than control mice which were not fed HN001 (Table 1). In contrast, only mice which received live HN001 had significantly higher gut mucosal antibody responses to cholera toxin than control mice (Table 1). There were no statistically significant differences in phagocytic or antibody responses when comparing between mice which received live or killed HN001. Neither live nor heat-killed HN001 had any significant effect on the numbers of leucocytes in blood samples, or on spleen wet weight (Table 2).

Dose–response effect of HN001 on enhancement of phagocytosis

In the dose–response study, cellular phagocytosis was selected as the most sensitive indicator of immune-enhancing properties of viable HN001 cells (Gill *et al.* 2000). Mice which had been fed 10^7, 10^9 or 10^{11} HN001 daily had significantly enhanced blood cell phagocytic capacity compared with control mice (Fig. 1(A)). Among the different treatment levels, mice which received 10^{11} HN001/d had significantly higher blood cell phagocytic responses than those which received 10^7 or 10^9/d. Mice which had been fed 10^9 or 10^{11} HN001 daily had significantly enhanced peritoneal cell phagocytic capacity compared to control mice (Fig. 1(B)); there were no significant differences in peritoneal cell phagocytic responses between the different treatment levels.

Discussion

Results from this present study have demonstrated that *L. rhamnosus* HN001, delivered orally as a viable probiotic supplement in a milk-based substrate, is able to enhance phagocytic capacity in mice. This confirms results of previous studies (Gill, 1998; Gill *et al.* 2000), however, an interesting further observation here was that heat-killed preparations of *L. rhamnosus* HN001 also enhanced phagocytic responses. While some reports have suggested that bacterial cell viability is a prerequisite to immune enhancement conferred by LAB (Salminen *et al.* 1998*b*), results here have indicated that at least phagocytosis can be enhanced using a similar dose of live or killed bacteria. Similarly, other reports have indicated that heat-killed *Lactobacillus* (Perdigon *et al.* 1986) or LAB cell wall fractions (Davidkova *et al.* 1992; Sasaki *et al.* 1994; de Ambrosini *et al.* 1996) can effectively stimulate the innate immune system in mice, following oral delivery. Solis-Pereyra *et al.* (1997) have suggested that LAB cell wall integrity, rather than cell viability *per se*, may be the critical factor in determining enhancement of cell function by

Table 1. Immunostimulatory effects of live or heat-killed *L. rhamnosus* HN001†

(Mean values with their standard errors for cell data and median values and 95 % confidence intervals for antibody data for eighteen mice per group)

	Phagocytically active cells (%)				Gut mucosal anti-cholera toxin antibody (units/ml mucus)	
	Peritoneal cells		Blood cells			
	Mean	SEM	Mean	SEM	Median	95 % CI
Controls	64·5	2·6	13·7	0·5	537	292, 1127
Live HN001	75·1***	1·3	20·9***	1·1	1937***	1115, 2522
Heat-killed HN001	71·9*	1·9	19·8**	0·5	812	481, 2358

Mean values were significantly different from those of the control group: * $P < 0{\cdot}05$, *** $P < 0{\cdot}001$.

† For details of diets and procedures, see p. 286.

Table 2. Absence of an effect of HN001 on leucocyte counts or spleen weight*†

(Mean values with their standard errors for eighteen mice per group)

	Leucocytes in peripheral blood samples ($n \times 10^6$/ml blood)		Spleen weight (mg)	
	Mean	SEM	Mean	SEM
Controls	3·7	0·7	100	3·4
Live HN001	4·0	0·4	98	2·1
Heat-killed HN001	4·7	1·0	101	3·0

* For details of diets and procedures, see p. 286.
† No significant treatment effects were detected.

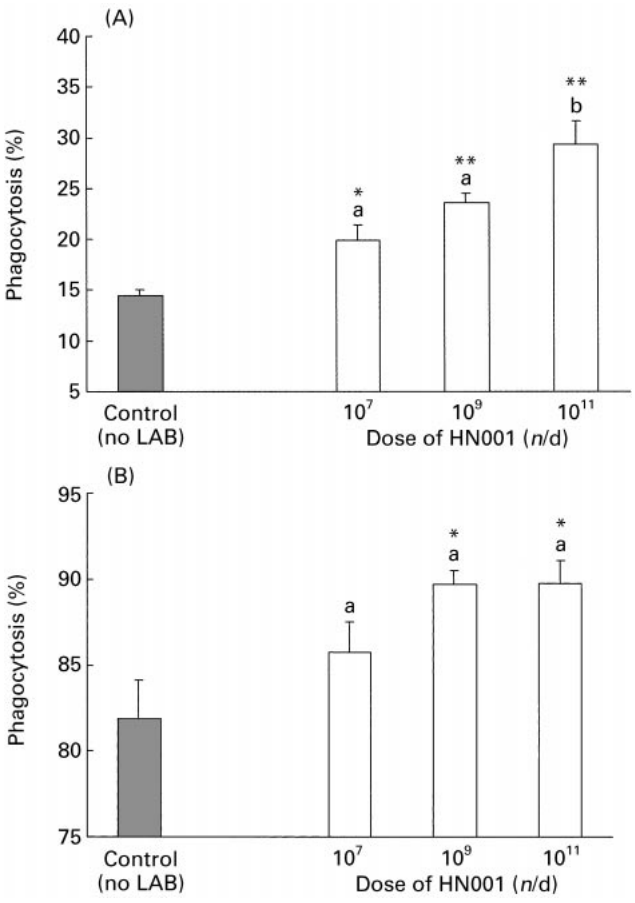

Fig. 1. Dose–response effect of HN001 on enhancement of leucocyte phagocytosis. (A), blood cell phagocytosis; (B), peritoneal cell phagocytosis. Mice were fed different doses of *L. rhamnosus* HN001 by oral gavage daily for 14 d. Phagocytic activity was determined by uptake of fluoresceinated *E. coli*. For details of diets and procedures, see p. 286. Values are the mean percentage of cells of each population showing phagocytic activity, with standard errors represented by vertical bars, for nine samples per group. Mean values were significantly greater than those of control mice: * $P < 0{\cdot}01$; ** $P < 0{\cdot}001$. [a,b]Mean values with unlike superscript letters were significantly different ($P < 0{\cdot}05$).

immunoregulatory LAB strains. It appears, therefore, that certain innate cellular immune responses (such as phagocytosis) may be responsive to bacterial components delivered in appropriate foodstuffs.

It was noteworthy in the present study that feeding of HN001, in either live or heat-killed form, was able to enhance phagocytic responses among two different leucocyte populations, namely blood cells and peritoneal cells. This observation is important, since the two tissue fluids are predominated by different leucocyte types, with polymorphonuclear cells representing the major phagocytes in the blood, while mononuclear cells (macrophages) are the predominant phagocytes in non-inflammatory peritoneal exudates (Hudson & Hay, 1992). Thus, dietary supplementation using LAB is able to promote immunoenhancing effects on phagocytic cells of different lineage and at different somatic sites. This systemic effect may be due to bacterial stimulation of secretory immunoregulatory leucocytes: a recent study by Nicaise *et al.* (1999) has demonstrated that colonisation of the murine gut with a normal LAB-rich microflora can regulate monokine production by both bone marrow- and spleen-derived phagocytes, suggesting that systemic effects of orally-delivered LAB might result from cytokine regulation of the immune system.

In contrast to the results obtained with cellular phagocytosis, the present study has indicated that live, but not heat-killed HN001 enhanced gut mucosal antibody responses to orally administered cholera toxin vaccine. This contrasts to the results of Portier *et al.* (1993) who demonstrated that both viable and heat-killed *Lactobacillus* preparations enhanced antibody responses to cholera toxin in mice, although their study utilised an intra-peritoneal route of vaccination, and they measured serum antibody responses. Our results indicate that viable *L. rhamnosus* HN001 cells are required to enhance this specific component of the immune system, suggesting that in this case, persistence of probiotic micro-organisms in the gut may be a prerequisite to enhancement of the mucosal immune system. It is known that immunoregulatory LAB stimulate the lymphoid follicles of the proximal small intestine (Peyer's patches) (Perdigon *et al.* 1994), and that mucosal plasma cells, derived from Peyer's patches, are primarily responsible for producing gut mucosal antibody. Furthermore, a recent study has shown that different preparations of LAB stimulate intestinal lymphoid foci and their accessory cells in different ways (Perdigon *et al.* 1999), lending further support to the notion that live forms of LAB can stimulate the specific compartment of the immune system differentially to killed forms.

In research concerning a potential food component that is destined to have a physiological effect, it is important to be able to optimise the dosage of the active component. In the case of immune enhancement, we have demonstrated here that a dose of 10^7 *L. rhamnosus* daily for 14 d was sufficient to enhance the phagocytic capacity of blood leucocytes in mice, confirming that this strain has potent immune-enhancing properties. Enhanced phagocytic cell function is one of the most commonly used indices of immune enhancement by LAB (Perdigon *et al.* 1986, 1988). The present study has demonstrated that increases in blood cell phagocytosis occurred without measurable changes in

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circulating lymphomyeloid cell numbers (blood leucocytes) or lymphomyeloid organ mass (spleen wet weight), suggesting that the immune-enhancing effects were due to a modulatory influence of HN001 on immune function, rather than general effects such as leucocytophilia or splenomegaly.

Interestingly, while a dose of 10^7 HN001/d was shown sufficient to enhance the phagocytic capacity of blood leucocytes, a minimum daily dose of 10^9 HN001 was found necessary to enhance the phagocytic capacity of peritoneal cells. Furthermore, while incrementally higher doses of HN001 imparted significantly greater levels of enhancement of blood cell phagocytosis, no significant increase in peritoneal cell phagocytosis was observed by increasing the daily dose from 10^9 to 10^{11}. Whether this observation represents a differential sensitivity of the respective cell types to the immune-enhancing effects of HN001 is unclear; however it is noteworthy that 10^9 micro-organisms/d has previously been utilised as an effective dose to enhance phagocytosis in peritoneal cell preparations in studies on other immune-enhancing strains of *Lactobacillus* in mice (Perdigon *et al.* 1986, 1988). It is likely, therefore, that enhancement of phagocytic cell function by *L. rhamnosus* HN001 is dose dependent, and that optimisation of the effective dose for human consumption would be warranted in clinical studies.

In summary, the present study has confirmed that *Lactobacillus rhamnosus* HN001 can modulate cellular and humoral immune functions in mice, following oral delivery. While heat-killed HN001 proved as effective as live cells in enhancing innate cellular immune function, only live HN001 enhanced specific gut mucosal antibody responses to orally administered cholera toxin vaccine. The conclusion of the present study is that the pleiotropic immune-enhancing properties of *L. rhamnosus* HN001 are best retained, in mice at least, by a dose of 10^9 viable micro-organisms/d.

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