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Oral administration of heat killed *Tsukamurella inchonensis* enhances immune responses and intestinal function in mice

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ABSTRACT: We studied the effects of heat-killed *Tsukamurella inchonensis* on immune parameters and intestinal structure in mice**.** Mice were treated with three doses of bacteria (5×10^7, 1×10^8 and 2×10^8 CFU/mouse) consecutively for seven days. Body weight, delayed type hypersensitivity response, relative organ weight, and haemagglutination titres were studied in different groups of animals. Villus height, villus width, villus/crypt ratio, crypt depth and goblet cell and intestinal epithelial lymphocyte density in villi were also determined. There was no significant increase in liver, spleen and kidney weights at any dose. Chicken red blood cell was used as a model antigen in the humoral and cellular immune response tests and *Tsukamurella inchonensis* elicited a significant ($P < 0.05$) increase in the delayed type hypersensitivity response at doses of 2×10^8 CFU/mouse. In the haemagglutination titres test, *Tsukamurella inchonensis* showed a modulatory effect at a dose of 2×10^8 CFU/mouse. There were clear increases in the height of villi and depth of crypts in all three treated groups, but no significant effects on villus/crypt ratio and villus width. Goblet cell density was increased significantly only in high dose-treated mice, while intestinal epithelial lymphocyte density was increased significantly in medium and high dose-treated mice. Overall, *Tsukamurella inchonensis* showed a stimulatory effect on immune functions and enhanced immune barrier function in the intestines of mice.

Keywords: immunomodulation; delayed type hypersensitivity; haemagglutination titre; intestine; villus; histology; liver; spleen; kidney

A number of aerobic *Actinomycetales* species, including *Gordonia bronchialis* (*G. bronchialis*), *Rhodococcus coprophilus* (*R. coprophilus*) and *Tsukamurella inchonensis* (*T. inchonensis*), have been described, which are closely related to mycobacteria, and which are capable of using subtly different adjuvant or immunomodulatory activities when injected as suspensions of killed bacilli (Tarres et al. 2012). It was found that in animal models, preparations of these species have been especially successful in preventing inflammation of the intima of arteries damaged with a balloon catheter (Stanford and Stanford 2012) and for prevention and treatment of spontaneous type 2 diabetes mellitus (Tarres et al. 2012).

Barrier function in the intestine is reflected in intestinal morphology. The heights of the villi and crypt depths are both important for intestinal function, and the size and surface area of the villi make up the area across which nutrients are taken up, with larger villi facilitating uptake. Crypts are thought to play a role through the secretion of enzymes that enable digestion and absorption, and increases in crypt depth reflect an increase in overall crypt cell number and an enhancement of digestive efficacy (Yang et al. 2009).

There are several factors that contribute to intestinal barrier integrity, such as the mucus coat and intraepithelial compartments of gut-associated lymphoid tissue (Khailova et al. 2009; Li et al. 2012).

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Mucus forms an important intestinal compartment which is secreted by intestinal mucus goblet cells and is known to have a highly dynamic matrix, largely consisting of mucin glycoprotein sheets that lubricate the transit of intestinal contents (Li et al. 2015).

The intestinal mucosa provides a protective host defence against the constant presence in the gut lumen of antigens from food and the normal microflora (Ohland and MacNaughton 2010).

Intestinal intraepithelial lymphocytes (IELs) are the first in line to come into contact with antigens present in the gut (Hershberg and Blumberg 2005; Cheroutre et al. 2011). They are located between the enterocytes in the epithelium above the basement membrane and are phenotypically and functionally distinct from lymphocytes in the underlying lamina propria or draining lymph nodes (Vega-Lopez et al. 1993; Rieger et al. 2015). IELs form a highly specialized lymphoid compartment in the intestine and are considered to play an important role in the regulation of mucosal immune responses (Li et al. 2012).

Some of the therapeutic uses of *T. inchonensis* include its use in the treatment of asthma and periodontal disease (Stanford and Stanford 2012). It is also used for the treatment of common colds, recurrent cold sores due to herpes simplex virus infection in man, and treatment of sweet itch and chronic obstructive pulmonary disease (heaves) in the horse (Stanford and Stanford 2012). However, the available evidence is not yet adequate to allow its use in clinical practice.

The objective of the present study was to study the effects of *T. inchonensis* on the immune system and intestinal function.

MATERIAL AND METHODS

Animals. This experimental study was approved by the Ethics Committee of the School of Veterinary Medicine, Tabriz University. Fourteen male suriyan mice (20–22 g) were provided by the Pasteur Institute of Tehran, Iran. The animals were bred and kept under standard laboratory conditions (temperature 25 ± 2 °C; photoperiod of 12 : 12 h). Commercial pellet diet and water were provided *ad libitum*.

Dosage. For the *in vivo* study, animals were divided into four groups (I–IV), each group comprising a minimum of 10 animals. Group I (control) received normal saline; Group II, bacterial suspension containing 5×10^7 CFU *T. inchonensis*/mouse; Group III, bacterial suspension containing 1×10^8 CFU *T. inchonensis*/mouse; and Group IV, bacterial suspension containing 2×10^8 CFU *T. inchonensis*/mouse. The doses were administered by gavage daily for seven consecutive days, the control animals receiving the same volume of normal saline.

Body weight and organ weight. The body weight of five mice was recorded 24 h after the last dose. The animals were then euthanized and the kidney, liver and spleen dissected out and weighed. Relative organ weight was calculated as the percentage ratio between [organ weight (g) per body weight (g)] × 100.

Evaluation of humoral immune functions. Five animals from each group treated in the above-described manner were challenged with 0.2 ml of 10% chicken red blood cells (CRBC), *i.p.* on the 10^{th} day after the initiation of the experiment. The parameters of humoral immunity that were studied in those animals are listed below.

Haemagglutinin titre assay. The haemagglutinin titre assay was carried out using the procedure of Bin-Hafeez et al. (2003). On the fifth day after immunisation with 0.2 ml of 10% CRBC, blood was gathered from the hearts of five mice for serum preparation. Serial two-fold dilutions of serum were made in 50 µl of phosphate-buffered saline (pH 7.2) in 96-well microtitre plates and mixed with 50 µl of 1% CRBC suspension in phosphate-buffered saline. After mixing, plates were kept at room temperature for 2 h. The antibody titre was designated as the highest serum dilution showing visible haemagglutination.

Evaluation of cellular immune function. For evaluation of cellular immune function, the delayed type hypersensitivity (DTH) response was evaluated in the bacteria-treated mice. Four groups of animals were treated as described above.

Delayed type of hypersensitivity response. The DTH response was confirmed using the method of Karthikumar et al. (2011). After bacterial treatment on day eight, five mice of each group were immunised by *i.p.* administration (2 ml) of 0.5×10^9 CRBC/ml/mouse. The day of immunisation was considered as Day 0. On Day 1 (24 h after immunisation), animals in all groups were challenged by subcutaneous administration of 0.2 ml of CRBC (0.025×10^9 CRBC/ml/mouse) into the right hind

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foot pad, while 0.2 ml of phosphate-buffered saline was administered in the left hind foot pad to serve as a control. The DTH response was measured at 48 h after the immunisation (Day 2) and was expressed as percentage increase in paw volume.

Histological and histomorphometrical analysis. Five animals from each group were euthanized on the 8th day after challenge with *T. inchonensis* by cervical dislocation and liver, kidney and 5 mm of the terminal segment of the descending duodenum of each animal was obtained, dissected and fixed in 10% buffered neutral formaldehyde. Then, tissue samples were processed using a standard paraffin embedding technique. Briefly, tissue samples were directly dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin wax. Thin sections (5–7 µm) were stained with haematoxylin and eosin (H-E) and examined under a light microscope for histological and histomorphometrical analysis. For histomorphometrical analysis, villus height and crypt depth was evaluated in 10 crypt units of five selected sections from each animal using a systematic random scheme, as recommended by Oliver-Sequeria et al. (2014). Villus height was measured from the tip to the crypt junction, and the crypt depth was calculated as the depth of the invagination between and beside villi using a calibrated ocular micrometer. The ratio of villus/crypt length was also calculated.

The number of IELs was assessed in five well-oriented different villi by counting the number of lymphocytes per 100 epithelial cells (enterocyte) in each villus, and calculating the mean. The predominant distribution (villous-base, villous-tip and villous body) and epithelial location (supranuclear and subnuclear) of the lymphocytes was noted. Only the lymphocytes above the basal membrane were assumed to be IELs (Memeo et al. 2005; Nasseri-Moghaddam et al. 2008). For the determination of goblet cell density in villi, five thin sections from each animal were stained using periodic acid Schiff and the number of periodic acid Schiff-positive cells per 100 epithelial cells was estimated.

Statistical analysis. All values are expressed as mean ± SEM and comparisons between the groups were made using one way analysis of Variance (ANOVA) and Tukey's post hoc test. A probability of < 0.05 was considered significant. The data were analysed using the SPSS statistical analysis system (SPSS software for windows release 22.0; SPSS Inc., Chicago, USA).

RESULTS

Effect of bacteria on body weight and relative organ weight

There was no effect of treatment with heat-killed *T. inchonensis* on body or organ weight (Table 1).

Table 1. Effect of heat killed *T. inchonensis* on body weight and relative organ weight (g)

Treatment	Dose (mg/kg)	Body weight	Spleen	Liver	Kidney
Group I (Control)	normal saline	26.84 ± 0.90	0.25 ± 0.01	4.99 ± 0.18	1.39 ± 0.05
Group II	5×10^7	26.71 ± 2.90	0.27 ± 0.03	5.54 ± 0.64	1.46 ± 0.16
Group III	1×10^8	29.08 ± 2.18	0.23 ± 0.02	4.82 ± 0.36	1.30 ± 0.09
Group IV	2×10^8	26.40 ± 1.77	0.26 ± 0.02	5.17 ± 0.40	1.44 ± 0.11

Each value is mean ± SEM of five individual observations

Effect of bacteria on humoral immunity parameters

The titre of the control was eight, while mice in the 2×10^8 CFU/mouse dose group showed a haemagglutinin titre value of 25.6, a significant increase ($P < 0.05$; Table 2).

Effect of bacteria on cell-mediated immunity parameters

Heat-killed bacteria at a dose of 2×10^8 CFU/mouse resulted in a significantly ($P < 0.05$) enhanced DTH response (Figure 1), compared to the control animals.

Table 2. Effect of different doses of heat-killed *T. inchonensis* (CFU/mouse) on haemagglutination titre (HT), using chicken red blood cells as an antigen in mice, 10 days pretreatment

T. inchonensis in diet	HT (mean ± SE)
2×10^8	25.6 ± 3.91*
10^8	10 ± 2
5×10^7	10.66 ± 2.6
Control	8 ± 0

Values are presented as mean ± SEM of five mice
*$P < 0.05$, significantly different from controls

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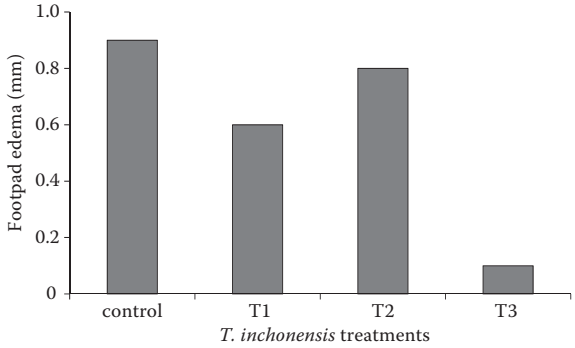

Figure 1. Effects of various doses of *T. inchonensis* on the delayed type hypersensitivity response in mice, compared with the control group

T1 dosed with 5×10^7 of bacteria, T2 given 1×10^8 of bacteria, T3 given 2×10^8 of bacteria

*$P < 0.05$ considered to be statistically significantly different from the control group

Histological analysis

The effects of oral administration of the heat-killed *T. inchonensis* on the morphology of the duodenal mucosal layer are shown in Figure 2. The administration of heat-killed *T. inchonensis* was associated with increased mucosal integrity and clarity compared to the control group.

The villus height and crypt depth, villus width and villus/crypt ratio in the upper small intestine of control and heat-killed *T. inchonensis*-treated mice on the day after the final treatment is shown in Table 3. Administration of heat-killed *T. inchonensis* resulted in significant changes to crypt depth and villus height compared to the control group, but there were no significant differences for villus width and villous/crypt ratio between the treated groups and the control group (Table 3). Histochemical and histomorphometrical analysis showed that oral administration of heat-killed *T. inchonensis* increased goblet cell density and the number of IELs in a dose-dependent manner. Statistical analysis showed that the numbers of goblet cells in the villi increased significantly ($P < 0.05$) only in the high dose-treated mice (Figure 3), while the distribution of IELs in the villus lamina epithelium was increased significantly ($P < 0.05$) in both medium and high dose-treated mice (Figure 4).

Table 3. Effect of various doses of *T. inchonensis* on villus height and crypt depth (μm), and villus/crypt ratio in mice

Groups	Villus height	Villus width	Crypt depth	Villus/ crypt ratio
Control	320 ± 3.53	92 ± 2.54	106 ± 1.41	3.03 ± 0.03
5×10^7	337 ± 2.54*	91.6 ± 3.17	111.8 ± 0.91*	3.01 ± 0.03
1×10^8	365 ± 3.50*	94 ± 2.91	116.2 ± 0.80*	3.14 ± 0.04
2×10^8	367 ± 3.00*	101 ± 1.87	121.4 ± 0.97*	3.02 ± 0.04

Each value is mean ± SEM of five individual observations

*$P < 0.05$ significantly different from control animals

DISCUSSION

In the present study, *T. inchonensis* showed an overall stimulatory effect on immune function in

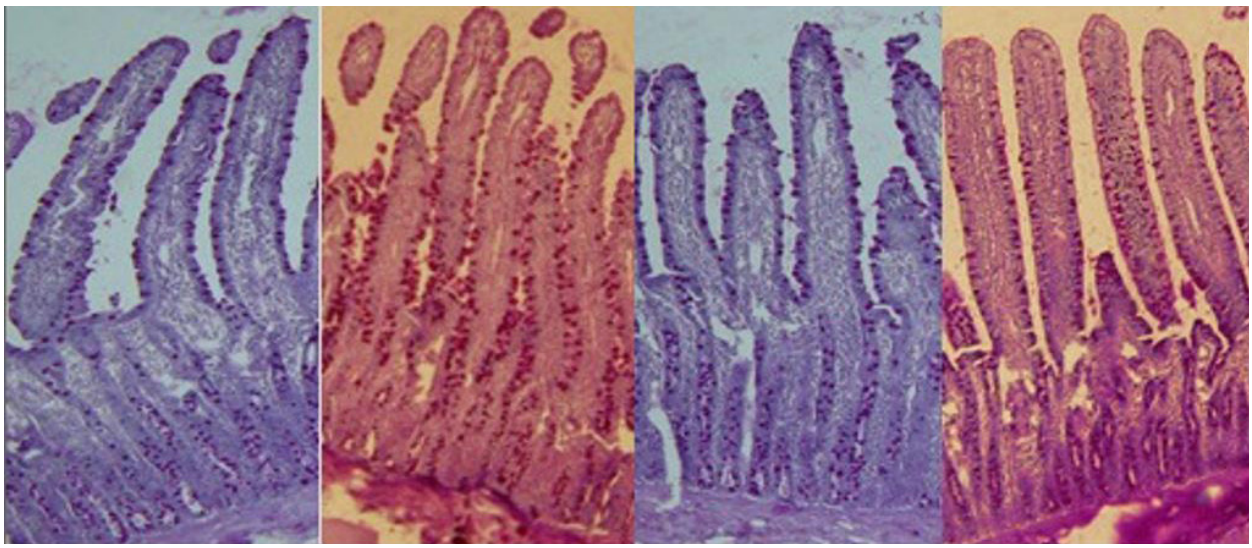

Figure 2. Photomicrographs of duodenums of mice administered phosphate-buffered saline (PBS) or different doses of heat-killed *T. inchonensis* (periodic acid Schiff, × 200)

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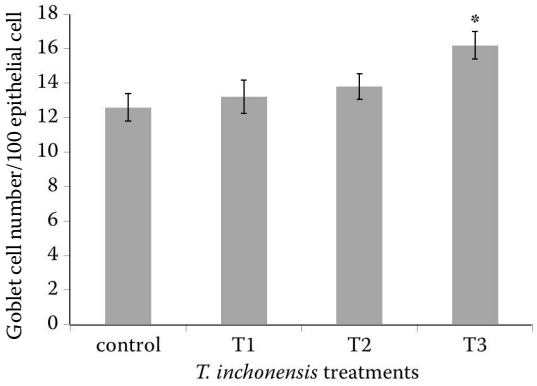

Figure 3. Effect of various doses of *T. inchonensis* on goblet cell density in the lamina epithelialis of duodenal villi in mice compared to the control group

T1 administered 5×10^7 of bacteria, T2 dosed with 1×10^8 of bacteria, T3 given 2×10^8 of bacteria

*$P < 0.05$ considered significantly different from control values

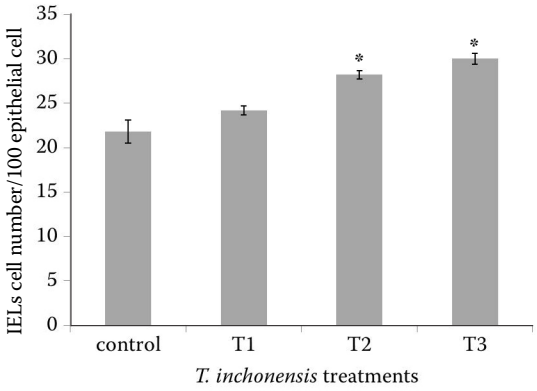

Figure 4. Effect of various doses of *T. inchonensis* on the distribution of intraepithelial lymphocytes (IELs) in the lamina epithelialis of duodenal villi in mice compared with a control group

T1 administered 5×10^7 of bacteria, T2 given 1×10^8 of bacteria, T3 given 2×10^8 of bacteria

*$P < 0.05$, significantly different from control group

mice. Stimulatory effects were detected on parameters of both humoral and cellular immunity. In the haemagglutinin titre test, the bacterium elicited an increased response at all the experimental doses, but this increase was only significant at the high dose of 2×10^8 CFU/mouse/day. This activity suggests that *T. inchonensis* is potentially able to affect and regulate immune reactions to "self" antigens including heat shock proteins as well as enhancing immunity to invasive bacterial challenges (Stanford et al. 2009). It appears that 2×10^8 CFU/mouse/day is the optimal humoral immunity-stimulating dose in mice.

The DTH response, indicative of cell-mediated immunity, was found to be the highest at the maximum dose of the bacterium (2×10^8 CFU/mouse/day) tested. The mechanism behind this elevated DTH during the cell-mediated immunity response could involve activation of sensitised T-lymphocytes. When challenged by antigen, they are changed to lymphoblasts and secrete substances such as pro-inflammatory lymphokines, attracting more scavenger cells to the site of the reaction (Sharififar et al. 2009). Several inflammatory processes have been suggested as possible mechanisms. For example, activation of complement, dissemination of mediators by activated mast cells, kinin, and reactive oxygen or nitrogen species from arachidonic acid metabolites (Karthikumar et al. 2011). An increase in the DTH response indicates that *T. inchonensis* has a stimulatory effect on lymphocytes and the accessory cell types required for the expression of the reaction (Thangakrishnakumari et al. 2013). After challenge with antigen, they are changed to lymphoblasts and secrete a variety of molecules also including pro-inflammatory lymphokines, which attract more scavenger cells to the site of reaction (Bafna and Mishra 2004). Similar results were shown for *M. vaccae* in different mouse strains (Rook and Stanford 1979).

T. inchonensis stimulated humoral, as well as cellular arms of the immune system. Previous studies have demonstrated the immunostimulatory effects of other Actinomycetales species. In a study performed in the UK, it was shown that priming with killed *M. vaccae* enhanced protective immune responses in laboratory mice (Rook and Stanford 1979). Bacillus Calmette Guerin enhances the cellular immune response but does not promote a shift of T cell responses from Th2 toward Th1 maturation (Stanford and Stanford 2012).

The data obtained here reveal that duodenum villus height and crypt depth were significantly higher in heat-killed *T. inchonensis*-treated mice killed 24 h after the last treatment. The heights of the finger-like intestinal villi correlate with the number of epithelial cells: taller villi are indicative of more mature epithelial cells and increased efficiency of intestinal absorption (Yang et al. 2009). We observed that mean duodenal villus heights increased after the administration of heat-killed *T. inchonensis* at all three doses tested. Similar results were observed by Yang et al. (2009) who administrated *Bifidobacterium* to mice. Heat-killed *T. inchonensis* significantly influ-

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enced crypt depth. Increased villus height can result from an increase in proliferation of crypt cells coupled with decreased apoptosis. The number of crypt cells with potential for cell division reflects the growth status of the intestinal membrane. The rate of crypt cell division determines crypt depth and can influence digestion in the small intestine (Kato et al. 1999). We found that mean crypt depths were increased after treatment with all three doses of heat-killed *T. inchonensis*, but there were no significant differences in the villus/crypt length ratio between mice fed with heat-killed *T. inchonensis* and the control group.

Our histomorphometrical results showed that oral administration of heat-killed *T. inchonensis* increased the number of mucus goblet cells and intraepithelial lymphocytes in the lamina epithelialis of duodenal villi. Mucus goblet cells are found along the entire length of the intestine and produce mucins to form a mucus layer. This layer provides protection by shielding the epithelium from potentially harmful antigens and molecules, while acting as a lubricant for intestinal motility (Ohland and MacNaughton 2010). The mucus is the first barrier that intestinal bacteria meet, and pathogens must penetrate it to reach the epithelial cells during infection (Madsen et al. 2001). It has been shown that some probiotic strains can stimulate protective responses by enhancing mucin secretion (Mack et al. 2003). Probiotics have also been shown to maintain the integrity of the mucosal barrier (Madsen et al. 2001; Stratiki et al. 2007). The results of this study showed that heat-killed *T. inchonensis* at a dose of 2×10^8 CFU/mouse/day could increase the resistance of the intestinal mucosal membranes by enhancing mucin secretion. Previous studies have suggested that one mechanism by which probiotics improve barrier function and exclude pathogens is through enhancement of mucus secretion (Fioramonti et al. 2003; Ohland and MacNaughton 2010).

IEL numbers also increased after oral administration of *T. inchonensis* in a dose-dependent manner. Studies addressing the normal range for IELs are few and some date back several decades (Ferguson et al. 1971; Mahadewa et al. 2002; Mino and Lauwers 2003; Biagi et al. 2004; Veress et al. 2004). In all of these studies there were different sample selections and methodologies, so no conclusion can be drawn with any certainty. It has been hypothesised that normal IEL numbers may be related not only to species, age, environmental exposures and ethnic background but also to the anatomical region of the gut. Nasseri-Moghaddam et al. (2008) suggested that IEL counts of less than 35/100 enterocytes found using immunohistochemistry methods and 34/100 enterocytes in H-E staining can be considered normal in human duodenum, while counts of more than 39 should be considered as elevated. However, they mentioned that regional differences in the normal upper limit for intraepithelial lymphocytes as well as changes over time should be considered when interpreting duodenal biopsies, although the H-E staining method seems adequate for clinical purposes.

Our data show that oral administration of *T. inchonensis* can increase the population of IELs to an optimal normal level. Under homeostatic conditions, IELs regulate the continuous turnover of epithelial cells by the elimination of infected epithelial cells and the control of epithelial repair (van Wijk and Cheroutre 2009). IELs are potentially the first immune cells to encounter exogenous antigens that enter via the oral route (Davies et al. 2004; Cheroutre et al. 2011). They are responsible for maintaining the integrity and homeostasis of the intestinal epithelial surface (van Wijk and Cheroutre 2009; Yu et al. 2012). It seems that medium and high doses of *T. inchonensis* have the potential to enhance the immune barrier function of the intestine by increasing IELs in the lamina propria. It has been shown that IELs profoundly regulate the induction of epithelial growth (Swamy et al. 2010). Klingspor et al. (2013) showed that administration of *Enterococcus faecium* in non-challenged piglets enhanced intestinal barrier function. Probiotic bacteria have been shown to promote endogenous host defence mechanisms (Isolauri et al. 2001).

Our observations show that heat-killed *T. inchonensis* exerts an effect on intestinal tract function through changes in intestinal morphology which correlate with the efficiency and barrier function of the intestine. The precise mechanisms through which heat-killed *T. inchonensis* can modulate intestinal function remain unknown, although it has been suggested that probiotic bacteria may accelerate crypt cell proliferation and growth and/or turnover of the intestinal epithelium.

The findings of the present study demonstrate that *T. inchonensis* has appreciable immunostimulatory activity. This study also shows that heat-killed *T. inchonensis* can elicit changes in the structure of the intestine mucosa in healthy suriyan

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mice. The heights of villi and depths of crypts were significantly increased, with inferred benefits for digestive efficiency and mucosal immunity. It is not possible at this juncture to single out the most effective immunostimulatory component of *T. inchonensis*. Its reported immunomodulatory effects warrant further study, for example as a potential immunostimulatory agent in immunocompromised individuals. We conclude that this strain can enhance intestinal function and immunity in mice, and may also be of benefit in human subjects.

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