

Effect of feeding micro-entrapped co-culture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on the immune response and protection of mice infected with *Salmonella typhimurium*

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Abstract – The present investigation evaluated the effectiveness of co-immobilized probiotic cultures in terms of survival, carrying out a challenge test and protection assay against the translocation of a specific pathogen, *Salmonella typhimurium*, using antibiotic-decontaminated mice models. The increase in the numbers of viable probiotic cultures in the intestinal tract, as achieved by feeding entrapped co-culture preparations, proved to be more effective at providing better protection to the host, as compared with the individual free cells. Our findings reinforced that administration of high numbers of probiotic cultures of lactobacilli and bifidobacteria, achieved by co-immobilization, reduced the translocation of enteropathogens such as *S. typhimurium* to extra-intestinal organs and thereby reduced the infection to the host. It was observed during the challenge studies that the survival rate of mice pre-treated with co-immobilized cultures was found to be a hundred percent as compared with 33 percent for the control group. A substantial increase in the circulating antibodies was observed by the 10th day of feeding in the groups of mice fed on co-immobilized culture preparations. Similarly, lysosomal enzyme activities in peritoneal macrophages from the groups of mice administered with co-immobilized culture were generally about 2.07 and 1.18 times higher than those of the control group for β -glucuronidase and β -galactosidase, respectively.

***Lactobacillus* / *Bifidobacterium* / micro-entrapped co-culture / immune response / protection**

摘要 – 嗜酸乳杆菌和两歧双歧杆菌共培养物的微胶囊对小鼠的免疫应答和鼠伤寒沙门氏菌的抗感染作用。本文通过对无特殊病原菌小鼠的刺激试验和保护分析, 研究和评价了嗜酸乳杆菌和两歧双歧杆菌 (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*) 共固定化培养物对抑制感染后的鼠伤寒沙门氏菌 (*Salmonella typhimurium*) 向其他器官的迁移作用。灌喂益生菌共培养物微胶囊试验组与其他灌喂单一益生菌试验组比较, 前者小鼠肠道中益生菌的数量明显增加, 并对宿主有很好的保护作用。研究证实了高菌数的共固定化乳杆菌和双歧杆菌培养物可以有效地减少肠病原体如沙门氏菌向外肠器官的迁移, 进而可以减少对宿主的感染。在刺激试验中还发现, 经共固定化益生菌培养物预处理后的小鼠肠道中益生菌的存活率为 100%, 对照组则为 33%, 而且经过 10 天的灌喂后, 在小鼠体内可以检测到大量的循环抗体。以 β - 葡糖苷酸酶和 β - 半乳糖苷酶表示巨噬细胞中溶酶体酶的活性, 试验证明灌喂共固定化益生菌培养物试验组中, 小鼠腹膜巨噬细胞中 β - 葡糖苷酸酶和 β - 半乳糖苷酶的活性分别是对照组的 2.07 和 1.18 倍。

乳杆菌 / 双歧杆菌 / 微胶囊化的培养物 / 免疫应答 / 机体保护

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Résumé – Effet de l'ingestion de cocultures micro-encapsulées de *Lactobacillus acidophilus* et *Bifidobacterium bifidum* sur la réponse immune et la protection des souris infectées par *Salmonella typhimurium*. La présente étude évalue l'efficacité de cultures probiotiques co-immobilisées en terme de survie ; challenge test et protection contre la translocation d'un pathogène spécifique, *Salmonella typhimurium*, chez des souris modèles décontaminées par antibiotiques. L'augmentation du nombre de bactéries probiotiques viables dans le tractus intestinal montrait qu'une meilleure protection était obtenue chez l'hôte avec l'ingestion de co-cultures encapsulées, comparées aux cultures individuelles non encapsulées. Ces résultats réaffirment que l'administration d'un nombre élevé de cultures co-immobilisées de probiotiques lactobacilles et bifidobactéries réduit la translocation vers les organes extra-intestinaux d'entéropathogènes tels que *Salmonella typhimurium* et réduit par là même l'infection chez l'hôte. Au cours de l'étude du challenge test, il a été observé que le taux de survie des souris pré-traitées avec des co-cultures immobilisées était de 100% contre 33% pour le témoin. Une augmentation substantielle des anticorps circulants était observée après 10 jours d'ingestion de préparations de cultures co-immobilisées. De même, les activités des enzymes lysosomales des macrophages péritonéaux était de façon générale plus élevées pour le groupe ayant reçu les cultures co-immobilisées par rapport au groupe témoin (2,07 à 1,18 fois plus, respectivement pour β -glucuronidase et β -galactosidase).

***Lactobacillus* / *Bifidobacterium* / culture co-immobilisée / réponse immune / protection**

1. INTRODUCTION

The microflora of the gut has a catalytic potential and has been implicated in both beneficial and detrimental effects on the health and well-being of the host. It is possible to prevent the deleterious effects and promote the beneficial effects of the intestinal microflora by manipulating its composition and metabolic activity. A good example is the introduction of live probiotic lactic acid bacteria into the intestinal tract [3]. Species of lactobacilli and bifidobacteria have been reported to be prominent members of the normal flora of the gastrointestinal tract throughout most of its length and for the life of the host [22]. Of these, bifidobacteria may comprise up to one-quarter of the gut flora of healthy adults. The two cultures have received greater attention because they reportedly exhibit a number of health benefits, including inhibitory action towards different enteric pathogens [5, 8, 10, 11, 15].

However, it has been frequently observed that the probiotic bacterial strains when ingested either become established at high or low population levels or be eliminated [6]. It has also been experienced that several bacterial strains given by oral supplementation to conventional mice were eliminated rapidly and only a few strains remained in the subdominant flora. Several

authors have termed this phenomenon as the barrier effect, which has also been recognized as bacterial antagonism, bacterial interference, colonization resistance [25] or competitive exclusion [14]. Thus, when the population level of a given strain was below 10^7 cfu·g⁻¹ fecal material, it did not play any significant role in the gut ecosystem due to the continuous renewal of intestinal contents.

Therefore, it is apparent that in order to provide any substantial health benefits, the cultures must reach the target site at a certain minimum level of living probiotic bacteria. Assuming a daily consumption of fermented milk equal to 100 g, a minimum level for probiotic bacteria of 10^6 cfu·g⁻¹ has been suggested in these products according to the daily efficient doses (10^8 cfu) as reported [27].

One way to improve probiotic cell counts in carrier medium is by physical entrapment of the organisms inside a polymeric matrix prior to their incorporation in the food product [26]. Previous work carried out in our laboratory has shown successful co-immobilization of probiotic cultures of lactobacilli and bifidobacteria in the most functional ratio of 2:1, respectively, for direct application to the host [12].

The studies relating to the mechanism underlying intestinal micro-ecology have so far relied extensively on in vitro models

that may not always reproduce the gut environment [9]. Many authors have since found small animals as *in vivo* systems to provide more relevant models for the study of microbial interactions. The two most common methods followed are the use of germ-free (GF) mice and specific pathogen-free (SPF) or antibiotic-decontaminated mice. These animal models were successfully used to study colonization resistance; a quantitative indicator that was measured in terms of the invading bacteria [18, 25]. The SPF mice were also used to study the bacterial translocation from the gastrointestinal tract in the immuno-compromised host [1, 16, 17, 28].

In view of the above, the present investigation was undertaken to examine the *in vivo* probiotic effect of feeding high numbers of cultures of *L. acidophilus* and *B. bifidum* as co-immobilized cells, and their protective effect.

2. MATERIALS AND METHODS

2.1. Handling of cultures

The strains of *Lactobacillus acidophilus* (NCDC 13) and *Bifidobacterium bifidum* (NCDC 255) were obtained from the National Collection of Dairy Cultures of the National Dairy Research Institute, Karnal, India. The cultures were maintained by weekly subculture in MRS and Yoshioka broths [29], respectively. For the differentiation of *Lactobacillus acidophilus* culture during isolation from co-culture preparations, Minimal Nutrient Agar with Salicin (MNAS) was used. On the other hand, *Bifidobacterium bifidum* was differentiated from the mixed culture using Modified MRS (MMRS) with selective agents such as nalidixic acid (0.15%), Cys HCl (5.0 g·L⁻¹) and LiCl₂ (30%). The cultures of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were tested for probiotic attributes such as resistance to low pH, bile tolerance, resistance to lysozyme, surface hydrophobicity and inhibition of enteropathogens, and were co-immobilized in calcium alginate beads [12].

2.2. Carrier medium for direct delivery of culture preparations

Sterilized skim milk was used as a carrier for oral administration of different culture preparations in free and micro-entrapped states to different groups of antibiotic-decontaminated mice (6 groups of 10 mice each). It was given at a 20 percent reconstitution in drinking water. The level of the two cultures individually was between 10¹⁰ and 10¹¹ cfu·100 mL⁻¹ with an average count of about 8 × 10¹⁰ of the carrier medium, in the case of free cells. At the same time, in the case of micro-entrapped cells, one gram of beads was suspended in 100 mL of the carrier medium. This resulted in approximately 2 × 10¹¹ cells 100 mL⁻¹ of *Lactobacillus acidophilus* and 1 × 10¹¹ cells 100 mL⁻¹ of *Bifidobacterium bifidum* in the carrier medium. The control group received 10 percent sterilized skim milk powder at 20 percent in the drinking water. In addition to that, all groups of mice were fed *ad libitum* with a balanced sterilized diet.

2.3. In vivo evaluation in animal models

Swiss albino mice (4–5 weeks old) weighing 20 to 30 g were obtained from the randomly-bred colony maintained at the Small Animal House of the National Dairy Research Institute, Karnal, India. Approved guidelines were followed for animal handling.

Each experiment was conducted on antibiotic-decontaminated mice (6 groups of 10 mice each). These groups were fed for 8 consecutive days before challenging: Group I (control), Group II (fed on free cells of NCDC 13), Group III (fed on free cells of NCDC 255), Group IV (fed on co-culture of free cells of NCDC 13 and NCDC 255), Group V (fed on entrapped co-culture 'A' beads; 2:1 ratio of NCDC 13 and NCDC 255), and Group VI (fed on freeze-dried 'A' beads). Each trial was conducted in duplicate and the data obtained were statistically analyzed.

For the antibiotic-decontamination process, mice were given Cefaxone (Ceftriaxone,

Lupin Labs Ltd., Mumbai, India) as a decontaminating agent at a level of $1 \text{ mg}\cdot\text{mL}^{-1}$ for two days ad libitum in their drinking water and the fecal counts were taken as day 0 and after 2 days to confirm the specific pathogen-free status, in this case *Salmonella typhimurium*. The antibiotic-decontaminated mice thus established were kept under barrier-sustained conditions in autoclaved polypropylene cages in a sterilized chamber. In addition to that, the mice were fed a sterilized diet and were given acidified water ($0.001 \text{ mol}\cdot\text{L}^{-1}$) after withdrawal of the carrier product.

The fecal samples were collected in sterile plastic containers and stored at 4°C for no longer than two hours before analysis. One gram (wet weight) of sample taken from the container was diluted in 9 mL of 0.1 percent sterile peptone and appropriate serial dilutions from each sample were plated on selective media as indicated above (see Sect. 2.1).

2.4. Challenge test on decontaminated mice

After the 8 days of consecutive feeding, the above 6 groups of mice were challenged with *Salmonella typhimurium* (MTCC-98, IMTECH, Chandigarh, India) cells in the smooth phase. The cells were repeatedly washed with saline solution before intubations using an oral catheter. For the translocation and antibody measurements, mice received 20LD_{50} each. This dose allowed the mice to survive for at least 15 days, while for the resistance assay (protection assay) doses of 40LD_{50} each were used.

2.4.1. Bacterial translocation

Two mice of each group were sacrificed by cervical dislocation on days 2, 4, 6, 8 and 10 after they were challenged with *S. typhimurium*, and their abdomens were swabbed with 70 percent ethyl alcohol. The skin and peritoneum were opened with sterile scissors and the spleen, liver and mesenteric lymph nodes (MLN) were removed aseptically. The numbers of viable bacteria were determined in the control and experimental groups.

To determine translocation to the mesenteric lymph nodes, the MLNs were excised and placed in 0.5 mL of brain heart infusion broth and homogenized with a Teflon grinder, and aliquots of 0.2 mL were plated on MacConkey's agar. The plates were incubated for 24 h at 37°C . Three milliliters of brain heart infusion (BHI) were added to the remaining 0.1 mL of MLN homogenate and incubated overnight at 37°C . Any growth was Gram-stained and sub-cultured on MacConkey's agar [2].

The number of viable bacteria in the liver and spleen were determined both in the control and experimental groups. At least two mice were sacrificed by cervical dislocation at different time intervals and the spleens and livers were removed aseptically. Organs were homogenized to a final volume of 5 mL in 0.1 percent peptone water with a Teflon homogenizer. The cell suspension was serially diluted in peptone water and plated in duplicate on MacConkey's agar plates. Lactose-negative colonies were counted after 24 to 48 h of incubation at 37°C . Confirmation of the identity of the isolates was done by biochemical tests [19, 21].

2.4.2. Evaluation of host immune response

2.4.2.1. Circulating antibodies

Mice of different groups were bled from the retro-orbital venous plexus. The sera were diluted and the antibody titers were determined against lactobacilli, bifidobacteria and salmonellae suspensions ($10^9 \text{ cfu}\cdot\text{mL}^{-1}$) using a tube agglutination test [21].

2.4.2.2. Antibodies from the intestinal fluid

The procedure for the collection of intestinal fluid was a modification of the method of Lin, Messhla and Watson [13] for the isolation of intestinal mucosal lymphoid cells. The small intestine was removed from each mouse from the stomach-duodenum junction and at the ileum-ascending colon junction. The intestinal contents were washed

out with 1.0 mL of cold phosphate buffer saline (PBS) with a pH of 7.2, centrifuged at $2000\times g$ for 30 min, and the supernatant was collected for determination of antibodies. Antibody titers were determined by diluting the intestinal fluid in PBS and agglutinating against lactobacilli, bifidobacteria and salmonellae as described above. Circulating and intestinal fluid antibodies were measured on days 2, 6, 10 and 14 post-challenge.

2.4.3. Determination of resistance/ protection assay

Treated and control groups of mice (Groups I to VI with 10 mice each) that had been fed for 8 consecutive days with co-immobilized probiotic cultures were challenged with 40 LD₅₀ of *S. typhimurium* and observed for survival up to 21 d. The daily death count was recorded to determine percent survival in each group.

2.4.4. Evaluation of macrophage activity

During the above study samples were also examined for the enzymatic activity and in vitro phagocytosis assay of peritoneal macrophages. Both tests were conducted on the 2nd, 5th and 8th days of feeding of culture preparations and a day after the challenge test on the groups of decontaminated mice.

2.4.4.1. Macrophage collection and culture

The mice were sacrificed by cervical dislocation and the peritoneal wash fluid was collected using 5.0 mL of Hank's medium (containing 100 units of penicillin and streptomycin per mL and 0.1 percent bovine serum albumin without glucose and stain) after gentle massage of the abdomen of the animals.

Portions of the peritoneal exudates containing 10^6 cells per milliliter were used for an in vitro phagocytosis assay. The rest of the exudates was distributed into 35-mm petri dishes and incubated in a humidified atmosphere of 5 percent CO₂: 95 percent air

for 2 h at 37 °C to allow the cells to get attached. Non-adherent cells were removed by washing three times with phosphate buffered saline. After being washed the cells were cultured in modified Hank's medium for 18 h.

The macrophage activity was measured by measuring the enzyme activity of β -glucuronidase and β -galactosidase cells [21].

2.4.4.2. β -Glucuronidase assay

β -Glucuronidase activity was determined on day 5 using the synthetic substrate p-nitrophenyl- β -D-glucuronide (Sigma) [25]. The assay was performed with 0.20 mL of cell supernatant, 0.25 mL pnPG (31.5 mg reagent and 100 μ L Triton X-100 in 100 mL of 0.05 mol·L⁻¹ sodium acetate – acetic acid buffer, pH 5.0) and 0.05 mL of buffer. The reaction mixture was incubated for 5 h at 37 °C and the reaction was stopped by adding 1.0 mL of 0.1 N NaOH. The absorbance was read at 410 nm in a spectrophotometer. The standard curve was prepared using different concentrations of p-nitrophenol (PNP). One unit of enzyme activity was defined as the nanomoles of PNP liberated from the substrate per hour per 10^6 cells [21].

2.4.4.3. β -Galactosidase assay

β -Galactosidase was assayed on day 5 using the synthetic substrate o-nitrophenyl- β -D-galactopyranoside (ONPG). A 0.2-mL sample was reacted with 1 mmol·L⁻¹ ONPG in 0.2 mol·L⁻¹ disodium phosphate 0.1 mol·L⁻¹ citric acid buffer, pH 3.8, in a final volume of 0.8 mL. The mixture was incubated for 5 h at 37 °C and the reaction was stopped by the addition of 0.8 mL of 0.5 mol·L⁻¹ Na₂CO₃. The absorbance was measured at 430 nm in a spectrophotometer. The standard curve was prepared using different concentrations of o-nitrophenol (ONP). One unit of enzyme activity was defined as the nanomoles of ONP liberated from the substrate per hour per 10^6 cells [21].

2.4.5. In vitro phagocytosis assay

To measure the phagocytic activity, aliquots of peritoneal macrophages (10^6 cells

per milliliter) were incubated for 15 min at 37 °C with the same volumes of bacterial suspension (10^7 cells of salmonellae per milliliter). The incubation was stopped in an ice-cold bath. The mixture was centrifuged for 5 min at $1500\times g$ and the sediment was observed under an oil-immersion lens in a phase contrast microscope. The percentage of macrophages with the ingested bacteria was estimated by counting a total of 200 cells.

2.5. Therapeutic effect

Two groups of SPF mice were used to separately study the therapeutic effect of feeding co-immobilized probiotic cultures. In this experiment, 10 mice of each group were first challenged with a dose of 20 LD₅₀ of *S. typhimurium*. After 48 h Group I was fed with free cells of a co-culture preparation of *L. acidophilus* and *B. bifidum* and Group II was fed with co-immobilized culture preparation of the two organisms. This feeding was continued for 7 consecutive days. During the feeding, the pattern of the growth of salmonellae in the MLN, liver and spleen were studied.

2.6. Statistical analysis

The data obtained in the above experiments were statistically analyzed [24] and means were compared for significant differences by Duncan's Multiple Range Test [7].

3. RESULTS AND DISCUSSION

Both the cultures *Lactobacillus acidophilus* (NCDC 13) and *Bifidobacterium bifidum* (NCDC 255) were selected based on a previous study conducted in our lab and had probiotic characteristics, as revealed by resistance to low pH levels (pH 1.0 to 3.0), resistance to the highest bile concentration of 2 percent for both the cultures even after 12 h of incubation, resistance to lysozyme (100 ppm), cell-surface hydrophobicity (SAT values of 0.8 and 0.5, respectively) and antibacterial activity [12].

3.1. Studies in antibiotic-decontaminated mice

To compare the effect of feeding various preparations of probiotic cultures on the resistance of mice to *S. typhimurium* and the immunomodulatory effects, antibiotic-decontaminated mice were used. The first step prior to the antibiotic treatment was the screening of different groups of mice for the presence of lactobacilli, bifidobacteria, salmonella and coliforms in their feces. The results obtained in the present study revealed the complete absence of salmonella and bifidobacteria, with very low lactobacilli in the feces of the mice. On the other hand, coliforms varied from log₁₀ 7.7 to 9.0 per gram feces.

The oral antibiotic treatment with cefaxone (ceftriaxone) resulted in complete elimination of these organisms from the intestinal tract of the mice, as evident from the two days of treatment. These mice were subjected to 8 days of consecutive feeding with different probiotic culture preparations to achieve their implantation prior to the challenge test with *S. typhimurium*.

The fecal counts taken on the 8th day of feeding indicated high counts of lactobacilli and bifidobacteria in groups V to VI, with the highest average log counts of 8.8 for lactobacilli (NCDC 13) and 8.3 for bifidobacteria (NCDC 255) in the group fed on micro-entrapped co-culture (Fig. 1). The statistical analysis of the data also revealed significant differences in not only the entrapped co-culture and control groups, but also with the co-culture of free cells at both the 5- and 1-percent levels. The oral administration of single culture preparations led to their survival in the host intestine as elaborated in Groups II and III. All the groups fed with probiotic preparations (Groups II to VI) showed the complete absence of coliforms in their feces, while the control group registered the reappearance of coliforms in the feces after the decontamination process.

3.2. Translocation assay using *S. typhimurium*

After feeding the different probiotic culture preparations for 8 consecutive days, the

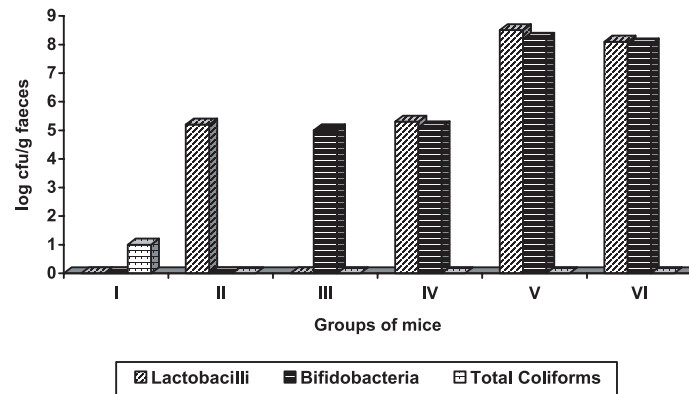


Figure 1. Implantation of probiotic cultures in antibiotic-decontaminated mice after 8 days of feeding.

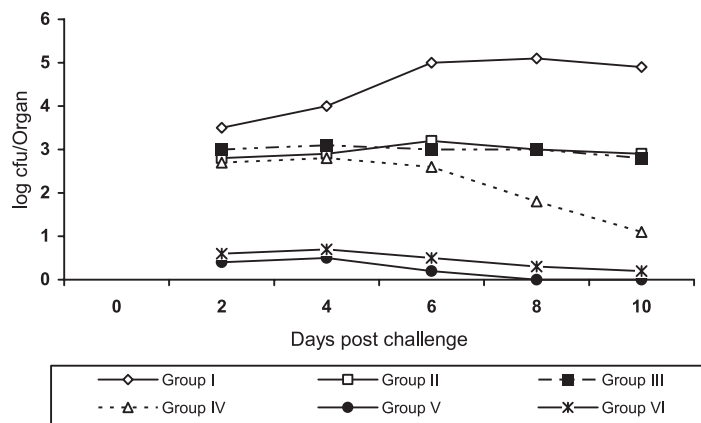


Figure 2. Viable counts of *Salmonella typhimurium* in MLN of mice fed with different culture combinations.

mice were orally challenged with 20 LD₅₀ of *S. typhimurium*. Viable bacteria in the mesenteric lymph nodes (MLN) were present from the 2nd day onwards, reaching the highest level on the 6th day (Fig. 2).

Similarly, the appearance of *S. typhimurium* in the liver and spleen was observed on the 2nd day, reaching the highest levels on the 6th and 8th days post-challenge in different groups (Figs. 3 and 4). It may further be seen from the data presented in the figures that the highest translocation was in the control group, which did not receive any

intake of probiotic cultures. It was also noted that the micro-entrapped co-culture provided better protection and the translocation of salmonellae was to the order of only 0.3 to 0.4 log₁₀ cfu per organ even after 6 days post-challenge.

It was also noticed that even the low cells of *S. typhimurium*, which got translocated to extra-intestinal sites, got eliminated on the 8th day from the MLN and spleen and on day 10 from the liver. The inferences were also supported by the least square analysis and the Duncan's multiple range

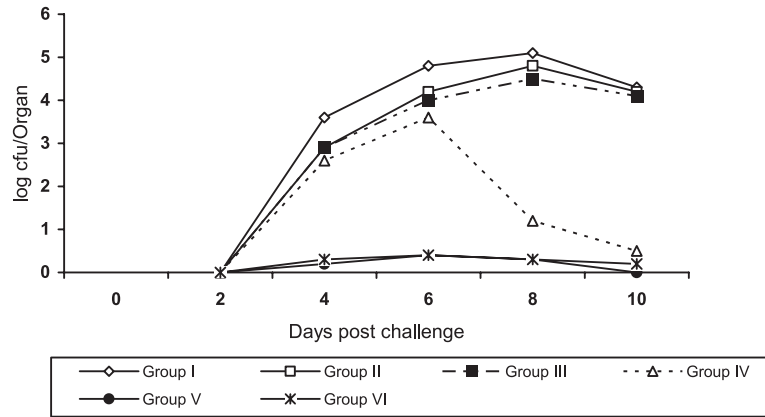


Figure 3. Viable counts of *Salmonella typhimurium* in liver of mice fed with different culture combinations.

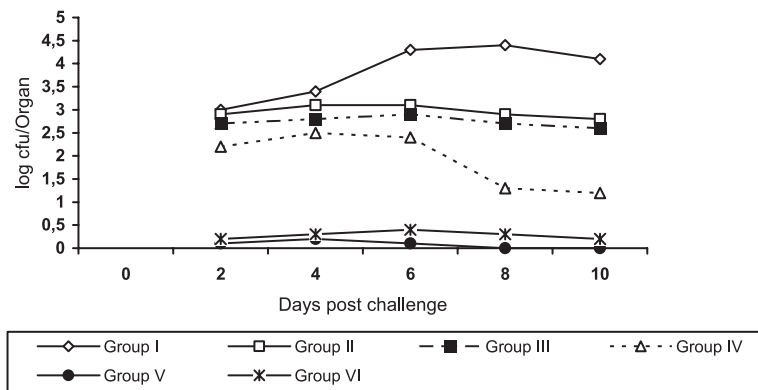


Figure 4. Viable counts of *Salmonella typhimurium* in spleen of mice fed with different culture combinations.

analysis of the data. Thus, our findings help to reinforce the hypothesis that the administration of high numbers of the co-immobilized probiotic strains NCDC 13 and NCDC 255 could help in reducing the translocation of enteropathogens such as *S. typhimurium* to extra-intestinal organs and thereby reduce the infection to the host. It was also brought out by the present study that the substantial increase in the numbers of viable probiotic cultures in the intestinal tract, as achieved by feeding entrapped co-culture, proved to be more effective at pro-

viding better protection to the host as compared with the free cells of probiotic cultures, both in single and associated form.

3.3. Evaluation of host immune response

The data on the levels of anti-salmonella antibodies from sera obtained on different days post-challenge are presented in Figure 5, and from intestinal fluid in Figure 6. Further perusal of the figures indicates that the titer on day 2 ranged from 1:10 for the control

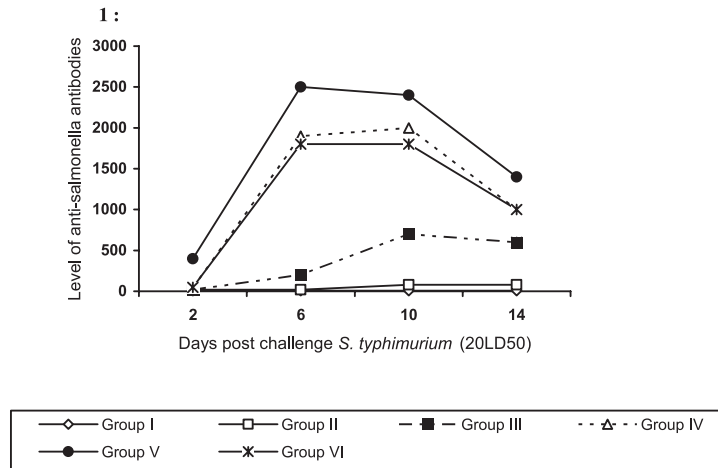


Figure 5. Levels of anti-*Salmonella typhimurium* circulating antibodies of mice fed with different culture combinations.

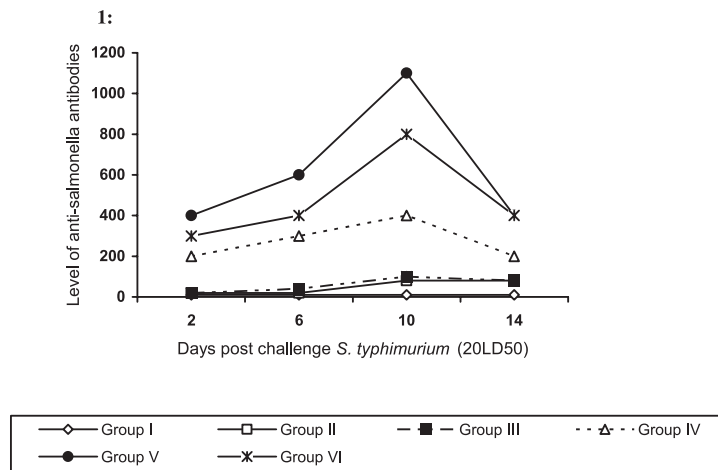


Figure 6. Levels of anti-*Salmonella typhimurium* intestinal antibodies of mice fed with different culture combinations.

group to 1:400 for Group V (the co-immobilized culture group) with intermediate values for the other groups. A substantial increase in the circulating antibodies was observed by the 6th day of feeding in Group V, which attained the highest titer of 1:2500. A similar trend in the evolution of the levels

of anti-salmonella circulating antibodies was observed for Group IV.

On the other hand, the level of anti-salmonella intestinal antibody titer was of a lower order. While the initial titer ranged from 1:30 to 1:400 for different groups, the highest response that could be achieved was

to the order of 1:1100 for Group V on the 10th day. The low levels of antibodies in the intestinal fluid as compared with circulating antibodies may be due to their digestion by the proteolytic enzymes produced as a consequence of increased inflammatory response [21].

In the present study, the titers of circulating antibodies towards lactobacilli and bifidobacteria of different groups were observed only in a few samples and that too at low levels of 1:10, when present. However, no antibodies to these two probiotic cultures were observed in the intestinal fluid. Low titers of anti-lactobacilli antibodies between 1:10 and 1:20, in the groups fed on milk fermented with *Lactobacillus casei* and *L. acidophilus*, were also reported by previous authors [21].

3.4. Determination of protection

The groups were challenged with 40 LD₅₀ *S. typhimurium* post-feeding for 8 d on different probiotic culture preparations and were observed for their survival for 21 d. Even after 21 d, the survival rate of mice pre-treated with co-cultures of free cells (Group IV) and co-immobilized cultures in 'A' beads (Group V) was found to be a hundred percent. However, in the case of freeze-dried co-immobilized culture beads (Group VI) the survival rate was 91.6 percent. The survival rate was further reduced to a level of 75 percent and 83.3 percent in single-culture groups fed with lactobacilli and bifidobacteria, respectively. In a similar study, the effect of oral treatment with *Bifidobacterium longum* Bb46 on intragastric challenge with *Salmonella typhimurium* was studied, and a higher survival rate (40%) was observed for probiotic-treated animals when compared with the control group [23].

Keeping in mind the kinetics of invasion of salmonellae in the control group, the effectiveness of previous feeding with different types of probiotic culture preparations for the protection of mice must be taken into account until day 9 post-challenge. After that time, the response to the pathogen was independent of the previous treatment. A previous report on these lines

[21] also reflected on the utility of considering the protection effect until day 7 post-challenge with *S. typhimurium*.

3.5. Therapeutic effect

In experiments carried out to study the therapeutic effects of different preparations of probiotic cultures, where mice were given these probiotic preparations after challenging them with *S. typhimurium*, the growth in the MLNs, liver and spleen showed no significant difference from the control group. This may agree with the earlier concept that antibodies secreted in response to the infection did not participate in displacing pathogens already bound to the host. Although there were other immunological factors that could protect the epithelial surface against bacterial interactions preventing pathogen colonization, they were not effective in the mixed fermented milk treatment after Salmonella challenge [21]. In another study with malnourished mice, it was reported that *L. casei* (combined with FOS), when given along with a re-nutrition diet, enhanced the immune response and increased resistance to certain pathogenic bacteria in the digestive tract [4].

3.6. Enzymatic activity in peritoneal macrophages

The term macrophage activation is widely used for an enhanced state of biological activity. Many parameters such as adherence, phagocytosis, bactericidal activity and biochemical characterization can be taken as indicative of the degree of macrophage activation [18].

In the present study, lysosomal enzyme activities and phagocytosis were taken as the primary parameters reflecting the degree of macrophage activation (Tab. I). It was observed that the enzyme activity of β -glucuronidase in peritoneal macrophages from the groups of mice administered with co-culture groups (IV, V and VI) were higher (40.47, 31.17 and 34.32, respectively) than that of the control group (19.47) after 5 d. These values were found to be significant based on comparing of means by Duncan's multiple range test. Similarly,

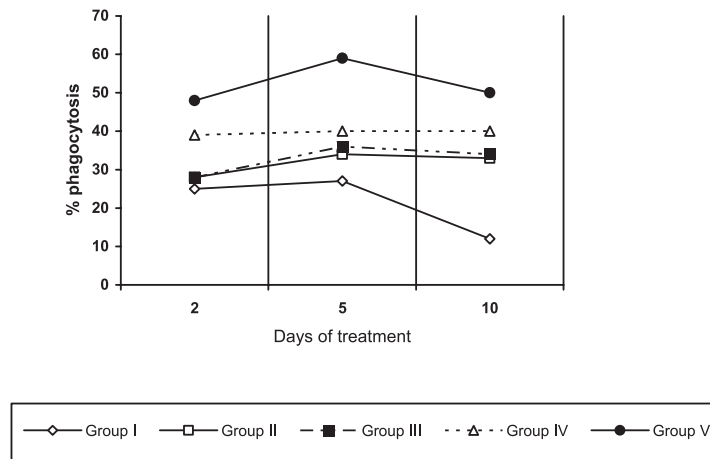


Figure 7. In vitro phagocytosis assay.

Table I. Enzyme activity as an indication of macrophage activity.

Groups	Mean enzyme activity \pm SD	
	β -Glucuronidase	β -Galactosidase
I	19.47 \pm 0.07	84.86 \pm 0.90
II	20.90 \pm 0.57	86.11 \pm 0.46
III	28.36 \pm 0.04	84.90 \pm 0.88
IV	40.47 \pm 0.94	100.60 \pm 0.20
V	31.17 \pm 0.53	93.59 \pm 0.26
VI	34.32 \pm 0.90	85.29 \pm 0.27

β -galactosidase activity of cultured peritoneal macrophages obtained from co-culture groups was also higher (100.60, 93.59 and 85.29, respectively) and was found to be statistically significant as compared with the control group (84.86). This indicates a better response in the case of co-culture groups; however, the reasons for the relatively higher values for the free co-culture group (Group IV) as compared with the co-immobilized groups (Group V and Group VI) could not be explained. In a previous study also, it was demonstrated that milk fermented with *L. casei* and *L. acidophilus* or

a mixture of both produced a remarkable effect on immunomodulation in the host [18].

3.6.1. Enhancement of phagocytic activity

In vitro phagocytic activity of peritoneal macrophages fed on different preparations of probiotic cultures showed a peak on the 5th day of administration with values three- to fourfold higher than that of control, and a further reduction in activity was found on day 8 (Fig. 7). The maximum phagocytic activity was observed in the groups that were fed with co-immobilized cultures. Earlier reports also demonstrated the immuno-stimulation of the host in terms of macrophage activation by feeding with lactobacilli, and the highest activity was obtained on the second to third day of administration of the fermented milk [20].

4. CONCLUSION

The present study reveals the effectiveness of micro-entrapped co-culture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* at exhibiting a protective effect in mice, including immune-response augmentation.

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