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Full Length Research Paper

Fluorescence investigation of discriminatory phagocytosis of live *Escherichia coli* and *Lactobacillus rhamnosus* by J774 Murine macrophage

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To investigate the phagocytic behavior of J774 murine macrophages in the presence of *Lactobacillus rhamnosus* and *Escherichia coli*, the bacteria were cultured and labeled with Fluorescein Isothiocyanate (FITC) and Rhodamine6G fluorescent dyes respectively and incubated with the macrophages. On ingestion, fluorescence micrographs of the culture were captured using fluorescein and Rhodamine filters respectively and processed using the *Openlab* software. The resultant images were synchronized with *Imagehopper2* and analyzed. The fluorescence micrographs revealed that Murine macrophages selectively internalized *E.coli* in the presence of the probiotic, *Lactobacillus rhamnosus.* The results suggest a molecular probiotic recognition mechanism in the mammalian immune system with potentially therapeutic applications and emphasize the increasing importance of fluorescence labeling methods in live imaging of in-vitro studies.

Key words: Discriminatory phagocytosis, phagocytosis assay, fluorescence microscopy.

INTRODUCTION

The imaging of live cells is a very important process in understanding both in-vitro and in-vivo cellular events. Several methods have been used, including ones that target organelles, specific proteins or metabolic products (Bereiter-Hann et al., 1983; Campbell and Drevets, 1991; Faulk and Lauf, 2001). Older methods have involved the use of fluorescent dyes, while novel ones have incorporated recombinant fluorescent proteins (Matz et al., 1999; McKinneys, 2009). These methods make it possible to observe cellular metabolism at the molecular level. Despite the numerous advantages, several challenges of using recombinant protein methods have been identified, necessitating not only the optimization of these methods but also an improvement on the traditional fluorescent staining techniques (Hanahan *et al.*, 1991; Kim et al., 2005). This research concentrated on studying the phagocytic behavior of J774 murine macrophage in the presence of *E.coli* and the probiotic *Lactobacillus rhamnosus* using improved staining techniques.

Macrophage phagocytosis is important in the initial innate immune response to bacterial infection. Many gram positive bacteria including species of *Clostridium*, *Listeria*, *Bacillus*, *Streptococcus*, *Staphylococcus* and *mycobacterium* have also been identified to evade the mammalian immune response (Pieters, 2008). More so, many species of this group are probiotic bacteria, having the ability to survive as commensals within the human body. Studies on the bacterial cell envelope indicate that the peptidoglycan coat of gram-positive bacteria may play a role in their pathogenicity or commensalism (Dziarski and Caputa, 2006). A Peptidoglycan recognition protein conserved from insects to human innate immune system has also been discovered (Kang et al., 1998).Thus the molecular architecture and chemical composition of bacterial of exopolymers may be a determinant in macrophage phagocytosis. This study would employ the use of fluorescence microscopy to investigate the relative efficiency of macrophage phagocytosis of gram-positive

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probiotic and gram-negative bacteria as a possible factor in bacterial infection and probiotic recognition.

MATERIALS AND METHODS

Reagents

The reagents were prepared as per manufacturers' instruction. Stock solutions were prepared from samples as delivered by manufacturer while working solutions were obtained by dilution with the stipulated solvent according to desired concentrations in line with available literature on previous works .These included Fluorescein 5 (6)-Isothiocyanate (FITC) and Rhodamine 6G both from *Sigma Aldreich* . The stock and working solutions were obtained by dissolving the dry samples of the dyes in the corresponding solvent. A 1 mg/ml stock solution of FITC was obtained by dissolving 1 mg of FITC in 1ml of anhydrous Dimethyl sulfoxide (DMSO). The working solution was prepared by subsequent dilution with 0.1M Sodium Hydrogen Carbonate ($NaHCO_3$) buffer at a PH of 9.0. Subsequent concentrations (l µg/ml and 2 µg/ml) were obtained by dilution factors.1 µl and 2µl of stock solution were made up with the diluent to 1ml each of working solution. The 0.1 M Sodium Hydrogen Carbonate buffer was prepared by dissolving 0.84 g of $NaHCO_3$ in 100 ml of distilled water. Drops of Hydrochloric acid (HCl) were added until the pH was 9.0.

A 1mg/ml stock solution of Rhodamine 6G was obtained by dissolving 1mg of Rhodamine 6G in 1ml of distilled water. The working concentrations (l µg/ml and 2 µg/ml) were obtained by making up 1 µl and 2µl of stock solution with Phosphate buffered Saline (PBS) to 1 ml each of working solution.

Bacteria and media

Escherichia coli HfrC and *Lactobacillus rhamnosus* strain GG were both obtained from Teesside University. *E. coli* was cultured overnight in Nutrient broth with shaking at 37°C while LGG was grown overnight in MRS broth with shaking at 37°C. The cell line used in this research, J774 murine marophage was cultured in macrophage culture medium containing 89% Dulbeco modified eagle medium (DMEM, Gilbco 31885), 10% Fetal bovine serum (Gilbco, 10270) and 1% Penicillin-Streptomycin solution (Gilbco, 10378016) and incubated at 37°C. Sub culturing of the macrophage was carried out on a three daily basis to ensure a confluent growth of not more than 80% and healthy cells.

Labelling *Lactobacillus* with Fitc

Lactobacillus rhamnosus (LGG) was labelled with FITC as adapted from Smith et al. (1998) and optimized for dye concentration and incubation period. 1 ml of overnight LGG culture was removed from the culture flask, put in an Eppendorf tube and centrifuged for 3 min at 12,000 g. The supernatant was discarded and the pellet was re-suspended in 1 ml of 1 µg/ml FITC working solution and incubated for 60 minutes at 25°C. The pellet was then centrifuged at 12,000 g for 1 min. The supernatant was removed and the pellet washed with 1 ml of Phosphate buffered saline and centrifuged again at 12,000 g for 1 min. This washing step was repeated five times until the pellet was clear. The pellet was then re-suspended in 1ml of phosphate buffered saline and the tube wrapped with Aluminium foil to protect it from direct light. The procedure was optimized to obtain clearer images by repeating with both concentrations of the dye at 15, 20, 25,30,35,40,45,50,55 and 60 min respectively.

Labelling *E. Coli* with Rhodamine 6g

Labelling was carried out with adaptation from Nishino and Yamaguchi (2002). 1 ml of overnight *E. coli* culture was removed from the culture flask, put in an Eppendorf tube and centrifuged for 1 min at 10,000 g.The supernatant was removed and the pellet was re-suspended in 1ml of 1µg/ml Rhodamine 6G working solution. Incubation was carried out at 37°C for 30 min. The cells were centrifuged again at 10,000 g for 1 min and the supernatant was removed. The pellet was washed with Phosphate buffered saline and centrifuged for 1 min. Washing was repeated for five times until the pellet was clear. Then the pellet was re-suspended in Phosphate buffered saline and covered with Aluminium foil to protect from light. The different concentrations of the dye (1 µg/ml and 2 µg/ml) were used as in the case of FITC to obtain an optimal concentration and incubation period.

Culturing of murine macrophages

The safety hood was sterilized by spraying with industrial motor spirit (IMS) and wiping with paper roll. Using a sterile pump, the medium in the culture flasks was removed.3ml of fresh medium was then added. The cell line number was written on the flask and a different passage number assigned. A sterile scraper was used to scour the culture and re-suspend the adhered cells in the fresh medium. Care was taken not to use the same scraper for different passage numbers. The culture was then mixed thoroughly by gentle pipetting to ensure an even dispersal of the cells prior to taking aliquots of 1ml each into two other flasks of same volume. Using a sterile pipette, the culture was transferred into a sterile flask and the volume made up to 5ml with fresh medium. Incubation was at 37°C and CO_2 tension of 5%. Sub-culturing was done on a three daily basis to ensure a confluence of not more than 80%.

Phagocytosis assay

The macrophages were observed under the light microscope for a confluence of ≥80% then the medium was removed. Fresh, phenol red-free macrophage culture medium containing DMEM, Penicillin/Streptomycin and Fetal bovine serum as described above was added, scraped and transferred onto four separate cover slips in a petri dish (Nunc 153066). 1ml each of was added to each dish to ensure adequate dispersal to cover the slip. The macrophages were allowed to adhere to the slips by replacing in the incubator at 37°C for one hour at a CO_2 tension of 5.0. After incubation, the slip was washed with 1 ml of Fetal bovine serum prior to inoculation to remove remaining media from the macrophages and increase accuracy of results.

The slip was removed with sterile forceps and mounted on

the *Nikon* fluorescence microscope. Microscopy was conducted with the Rhodamine and Fluorescein filters respectively. A bright field image of the macrophages was captured with x60 objective (oil immersion) to note the shape and size prior to inoculation of bacteria. Using a micropipette, exactly 10 µl of the Rhodamine 6G-stained *E.coli* was dropped vertically on the slip and allowed to spread evenly over the macrophages. Similarly, 10 µl of FITC-stained LGG was dropped on the slide. The Rhodamine and Fluorescein filters were used to capture the fluorescence images alternatively after 3 min of inoculation using the *Open lab* application of *Prism 4* software. The x60 oil immersion objective was combined with a x100 plan-fluor objective. Subsequent images were captured at 2 minute intervals, that is, 5, 7, 9,11,13,15 and 17 minutes respectively. Both fluorescent images were saved in TIFF format while changes in shape of the macrophages and visible ingestion of bacteria was noted and recorded accordingly. The captured images were converted on the *Image hopper 2* software and the corresponding colours of the filters were applied. For the image of the combined ingestion of *E. coli* and LGG, the two results were synchronized with the same software to illustrate actual internalization of bacteria in the endosome.

RESULTS AND DISCUSSION

In the fluorescence labelling experiments, one factor that contributed to the final quality of the fluorescence and image was the incubation time. This was observed with both Rhodamine 6G and FITC. The best quality for FITC was obtained at a concentration of 1µg/ml and45 minutes incubation (Figure 2), while the optimal concentration and incubation time for Rhodamine 6G was 1µg/ml and 30 minutes respectively (See Figure 1).

Longer exposure to the dye did not yield better images, while higher concentrations of both dyes led to greater background noise. Another factor that enhanced the uptake of the dyes was an additional centrifugation step after the incubation. This probably agitated the bacteria and rendered the cell envelopes more permeable to the dyes.

On incubation with the macrophages, visible ingestion commenced at 2 min. The changes in shape can be seen by comparing Figure 3 and Figure 6. Only the Rhodamine-stained, oblong shaped *E. coli* were internalized in the endosome, showing a comparative preference of phagocytosis (Figure 6). The FITC-stained probiotic can be seen as short dashes and chains around the macrophages (Figure 5). This observation was also made using the Rhodamine filter (Figure 4). The green stains on the surface of the macrophages were merely artefacts of the FITC preparation as no defined shapes were observed (Figure 7). The phenomenon of probiotic recognition and selective phagocytosis appears to differ in mechanism from the immune evasion strategies deployed by pathogenic bacteria described in previous studies. Certain pathogenic bacteria produce toxins that impede the movement of the macrophage towards the site of infection. *Staphylococcus aureus* produces an array of toxins to suppress macrophage chemotaxis and can often lead to lysosomal discharge into the cell's cytoplasm (Haas et al., 2005). *Clostridium perfringens ø* toxin has been demonstrated to retard macrophage chemotaxis (McDonel, 1980).Attenuation of human neutrophil migration by uropathogenic *E. coli* has also been reported (Loughman and Hunstad, 2011). These mechanisms have not been discovered in probiotic bacteria.

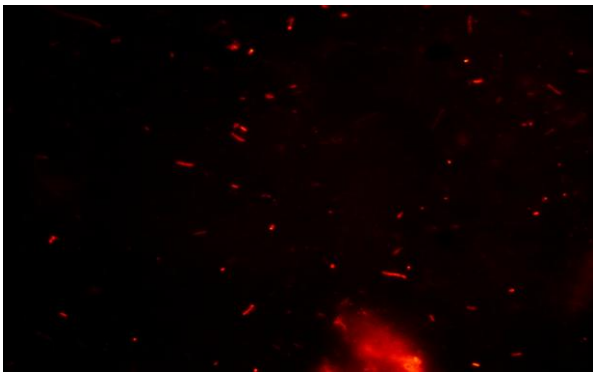

Figure 1. Fluorescence micrograph of Rhodamine 6G - stained *E. coli* (x6000).

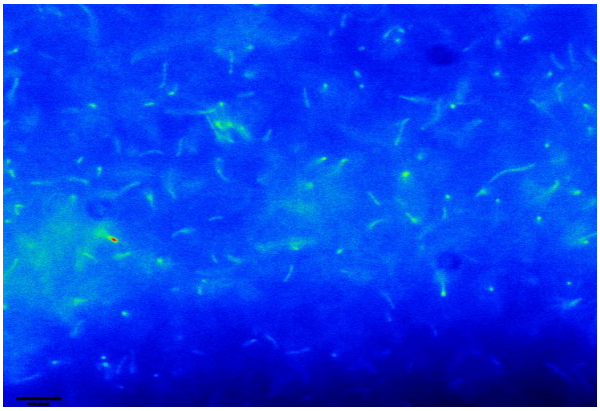

Figure 2. Fluorescence micrograph of FITC-stained *Lactobacillus rhamnosus* (X 6000).

Another strategy deployed by pathogenic bacteria is to resist or avoid engulfment by the macrophage. Since engulfment is facilitated by the presence of specific macromolecules on the bacterial cell envelope and cellular surface structures. Staphylococcus aureus evades the opsonisation stage by producing staphylokinase, an anti-opsonin. Studies by Laarman et al. (2010) showed that Staphylokinase activates human plasminogen into plasmin at the bacterial surface, leading to serine protease degradation of two major opsonins,

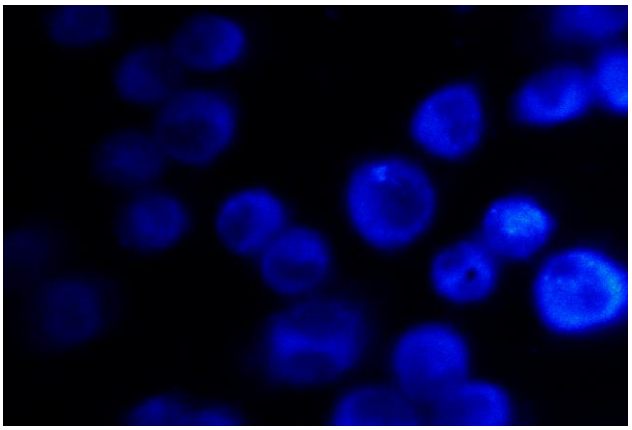

Figure 3. J774Murine macrophages prior to ingestion (X 6000). Note the smooth cell membrane prior to interaction with the bacteria.

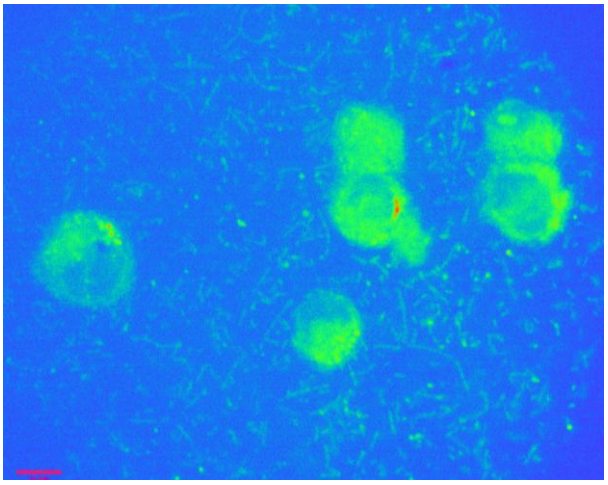

Figure 5. Fluorescence micrograph of combined phagocytosis using Fluorescein filter (x 6000). The probiotic bacteria can be seen as rather long dashes and chains of rod-shaped bacteria.

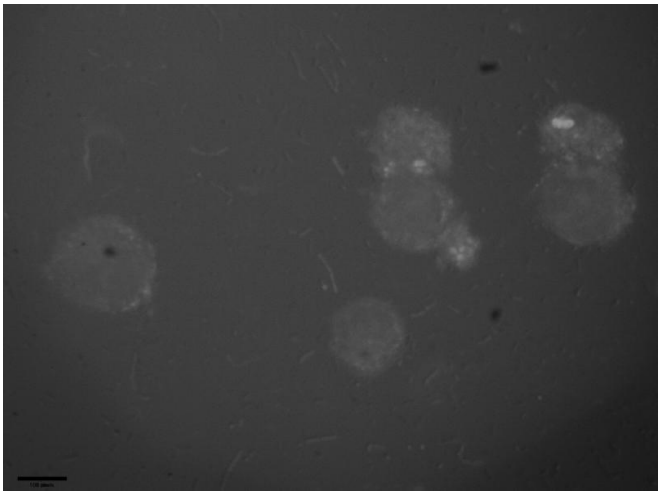

Figure 4. Fluorescence micrograph of combined phagocytosis using Rhodamine filter (X 6000). The changes in shape are clearly observable as the macrophages internalize bacteria.

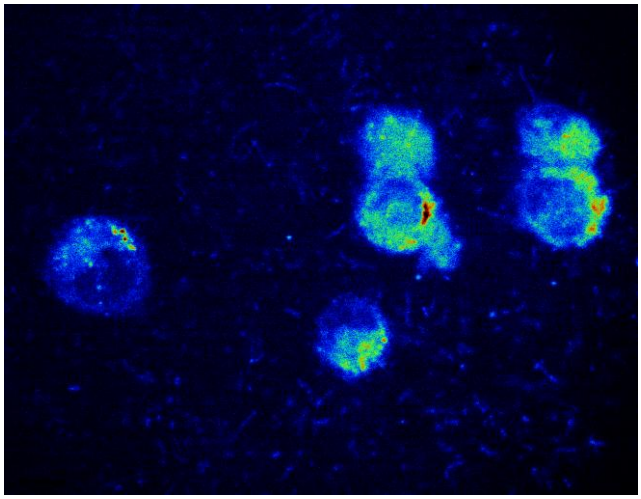

Figure 6. Synchronized image showing Rhodamine-stained *E. coli* in the endosome (X 6000).

Immunoglobin G (IgG) and human C3b.Species of *Streptococcus* produce M protein on their fimbriae to prevent adhesion to the surface of the macrophage, thus preventing engulfment (Vidova et al., 2009). They also produce hyaluronic acid capsule to evade recognition (Jong et al., 2007; Hymass et al., 2010). *Bacillus anthracis* produces poly D-glutamate capsule that makes macrophage engulfment difficult (Abeyta et al., 2003). A similar capsular polysaccharide is produced by *Streptococcus pneumonia*, *Traponema pallidum* and *Escherichia coli*. Smooth strains of *E .coli* possess the O-antigen while strains that produce the acid polysaccharide possess the K-antigen (De Verdier et al., 2012). *Staphylococcus aureus* produces protein A that inhibits the opsonisation stage of phagocytosis by effectively blocking the Fc portion of Immunoglobin molecules (Laarman et al., 2010). It also produces *coagulase* enzyme that conceals ligands necessary for macrophage interaction.

Another mechanism by which pathogenic bacteria evade phagocytosis is the prevention of phagosome formation. *Mycobacteria* inhibit plasma membrane fusion by interactions with the extrinsic protein Porin; thus preventing the compartmentalization of the pathogen (Buchwalow et al., 1997; De Chastellier and Thilo, 2002) while *Legionella pneumophilia* survives the stage of

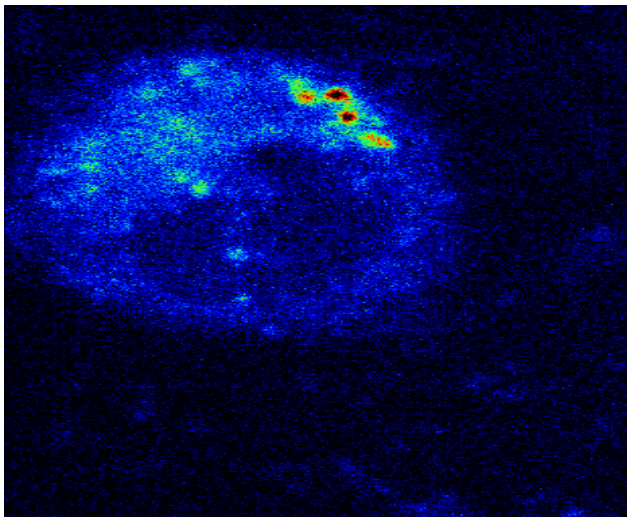

Figure 7. Enlarged image of macrophage (X 24,000) showing internalized Rhodamine-stained *E. coli* surrounded by phagosome membrane.

phagocytosis by inhibiting the formation of a phagolysosome and lysosome by produsing a protein kinase that phosphorylates host cell tubulin and phosphstidylinositol. Although it can be ingested, it remains and reproduces in a phagosome which is unable to fuse with lysosomes (Saha et al., 1989).Similar strategies are employed by *M. bovis* (Nguyem et al., 2005).

Certain bacteria can however survive in the phagolysosome. Species of Mycobacterium have been noted to survive in phagolysosomes. Most produce a hydrophobic cell wall constituted by myocolic acids and are resistant to lysosomal enzymes. Good examples include *M. tuberculosis* and *M. leprae (*Norman et al., 1994*)*. *Brucela abortus* also has lipopolysacharides that modulate lytic enzymes. *Riketsia* escapes from the phagosome by producing the enzyme phospholipase A, which disrupts the membrane of the phagolysosome, enabling escape. Similarly, *Streptococcus neoformans* escapes from the phagosome (Alvarez and Casaderal, 2006).*Listeria monocytogens* and *Shigella* possess identical strategies to survive after engulfment in the phagosome. *L. monocytogens* produces Lysteriolysin O and Phospholipase C to rupture the phagosome and initiate actin polymerization in the macrophage (Goldfine et al., 1993; Dancs et al., 2002). The resultant microfilaments enhance its mobility within the cell. *Shigella* releases lytic enzymes to secure its early escape from the phagosome and similarly induces the polymerization of acting filaments (Engelkirk and Duben-Engerlik, 2008). None of these mechanisms has been identified in probiotic bacteria.

Killing or damaging of phagocytes to prevent ingestion has been observed in most pathogenic bacteria. Some produce lethal enzymes while others produce toxins that result in cell death or extensive damage. Prior to ingestion, gram-positive pathogenic bacteria are known to secrete enzymes that perforate the plasma membrane, thereby lysing the cells. Species of *Streptococcus* produce Streptolysin. Cholesterol binding of Streptolysin in membranes has been demonstrated to lead to explosion of lysosomal granules and consequent release of autolytic enzymes into the cell cytoplasm (Timmer et al., 2009). Species of *Staphylococcus* are known to produce lethal leukocidin which not only affects cell proliferative ability, but also ruptures the cell membrane and results in lysosomal discharge and cell death (Bownik, 2006).

Certain extracellular proteins act as toxins and inhibit phagocytosis. *Pseudomonas aeruginosa* produces Exotoxin A, which is poisonous to macrophages (Vouloux et al., 2000). Anthrax toxin EF and Pertusis toxin AC are adenylate cyclase enzymes that challenge the macrophages by destabilizing the cell energy flux through the wasting of ATP reserves necessary for bacterial ingestion by the macrophages (Lepla, 1982).

Some pathogenic bacteria have developed killing strategies that are only employed after engulfment has taken place. Such bacteria survive in the phagosome and secrete toxins that pass through the phagosome membrane and induce the release of autolytic enzymes by the macrophage, leading to its death. *Mycobacterium, Lysteria* and *Brucella* are suspected to use this mechanism (Engelkirk and Duben-Engerlik, 2008).

The phagocytic adaptation of the probiotic bacteria to the mammalian macrophages is evident in the assay results. The molecular basis for this behaviour however may lie in the phage transformability of lactic acid bacteria (LAB), a group to which LGG belongs. It has been discovered that most lactic acid bacteria are Gram-positives with low G+C DNA content. This makes them easily susceptible to horizontal gene transfer from phage infections and subsequently evolved compatibility with the mammalian immune system. Similarly organized lysogeny modules in temperate *Siphoviridae* phages and lysogeny-related genes from Gram-positive bacteria have been identified (Lucchini et al., 1999) .

Ventura et al. (2003) further emphasized the relationship between the genome of lactic acid bacteria and their comensal relationships by comparative genomics and transcription analysis of *Lactobacillus johnsonii* NCC 533.It was discovered that a significant portion of the total genome was composed of phage genome remnants, a pointer to a possible horizontal gene transfer in the evolutionary history of probiotic bacteria.Subsequent research on phage-encoded Integrase and Endolysin in *Streptococcus gordonii* has provided evidence in support of the mechanism (Van der Ploeg, 2008).

Conclusion

There is a remarkable preference for *E. coli* as against probiotic *Lactobacillus rhamnosus* GG in phagocytosis by J774 murine macrophages. The results in this work suggest molecular probiotic recognition and discrimination mechanisms in the mammalian immune system .There is also a possibility of a phagocyte evasion strategy in probiotic bacteria, significantly different from the ones deployed by pathogenic microbes. Elucidation of this mechanism could prove to be a giant step in developing new therapeutic uses of probiotic bacteria. The design of selective therapeutics that can affect the pathogenic and potentially pathogenic bacteria on the basis of such recognition elements would make it possible to target pathogens without harming probiotic bacteria.

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