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Short Communication

Adherence and internalisation of *Lactococcus lactis* M4 towards human colorectal cancer cell line, Caco-2

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ABSTRACT

Aims: *Lactococcus lactis* is a non-colonizing, generally-regarded as safe (GRAS) lactic acid bacteria that has been frequently studied as a potential vector for bactofection. To mediate bactofection, a series of interaction between the bacteria and the host cell needs to occur. This study aims to investigate the *in vitro* bacterial-cell interaction between a locally-isolated *L. lactis* M4 strain with human colorectal cancer line, Caco-2.
Methodology and results: Bacterial interaction was evaluated via adherence and internalisation assays. A 250:1 ratio of bacteria to cancer cell was selected as the optimum multiplicity of infection for all assays. After 2 h, *L. lactis* M4 was able to adhere to and internalise into Caco-2 cells at comparable rates to commercial strains *L. lactis* NZ9000 and MG1363.
Conclusion, significance and impact of study: Findings from this study showed that this strain has similar interaction properties with the commercial strains and would make a promising candidate for future bactofection studies and development of bacteria-mediated DNA vaccination against various diseases.

Keywords: Lactococcus lactis, adherence, internalisation, Caco-2

INTRODUCTION

Bacteria-mediated delivery of plasmid DNA, or bactofection, is an effective approach for achieving direct gene transfer into target host. It involves a series of bacteria-host cell interactions such as adherence and internalisation of bacterial vector into host cell. Following internalisation, bacterial vector will be lysed, causing the release of plasmid DNA, which may get transferred into the host nucleus, where transcription and expression take place (Pálffy *et al.*, 2006). Bactofection is regarded advantageous in terms of its simplicity (Fu *et al.*, 2005) and gene transfer selectivity (Michael *et al.*, 2004), which can be coupled with other genetic technologies for DNA vaccination against microbial pathogens and a wide range of diseases, including cancer. However, not all bacteria-host cell interaction will result in cellular entry and DNA transfer. Various studies have demonstrated bactofection using live, attenuated bacteria such as *Salmonella, Listeria*, and *Pasteurella* (Jiang *et al.*, 2017; Kamal *et al.*, 2017; Sinha *et al.*, 2017). These highly invasive bacteria are efficient at gaining cellular entry and bactofection, but significant concerns have been raised over the possibility of these bacteria to regain their pathogenicity. Therefore, a safer bacterial vector is greatly needed as an alternative.

Food-grade lactic acid bacteria (LAB) such as *Lactococcus* and *Lactobacillus* have been proposed as suitable candidates for bactofection. Among the LAB family, *Lactococcus lactis* is one of the most well-characterised bacteria (Bolotin *et al.*, 2001) and is generally-regarded as safe (GRAS). Both *Lactococcus* and *Lactobacillus* have been identified to display probiotic potential (Kimoto *et al.*, 1999) and would make excellent candidates for oral vaccination strategies, but unlike *Lactobacillus* spp., *L. lactis* does not naturally colonize the human gastrointestinal tract. This decreases the possibility of long-term gut colonization, which could result

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in uncontrolled spread of the modified strain in a healthy individual. Furthermore, abundant studies have been done to promote the potential role of *L. lactis* as protein factory and vector for oral DNA vaccination. In one study, Steidler *et al.* (2000) reported the use of a modified *L. lactis* secreting interleukin-10 (IL-10) for the treatment of inflammatory diseases, which was followed by a phase I clinical trial and a successful method for biological containment of the genetically-modified organism (Steidler *et al.*, 2000; Braat *et al.*, 2006). Meanwhile, in the field of cancer research, Zhang and colleagues (2016) demonstrated the inhibition of colorectal cancer cell proliferation and migration using recombinant *L. lactis* secreting metastasis-suppressing peptide, KiSS1 in HT-29 cells.

Previously, a novel *L. lactis* strain M4 was successfully isolated from a local cow's milk and was characterized (Noreen *et al.*, 2011). Due to the promising potential applications of *L. lactis*, it is of great interest to determine whether this Malaysian strain is capable of displaying bacteria-cell interaction comparable with currently available commercial strains. In the present study, we evaluated *in vitro* interaction of the locally-isolated *L. lactis* strain M4 with human colorectal cancer cell line, Caco-2, based on bacterial adherence and internalisation abilities.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactococcus lactis M4 used in this study was a locally isolated strain by Noreen *et al.* (2011). On the other hand, commercial *L. lactis* strains NZ9000 and MG1363 were obtained from Mobitech GmBH, Germany. All strains were cultured at 30 °C, without aeration, on GM17 agar and broth consisting of M17 media (Merck, Germany) with 0.5% (v/v) glucose.

Cell culture

Human colorectal cancer cell line Caco-2 (ATCC, USA) was maintained in RPMI-1640 medium (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS, Tico Europe) in 5% CO_2 at 37 °C.

Cell viability assessment

Caco-2 cell viability was evaluated using the Trypan blue exclusion method (Strober, 1997). Cells were incubated with 0.25% (w/v) Trypsin-EDTA (Nacalai Tesque, Japan) for 5 min at 37 °C and trypsin was immediately deactivated with RPMI-1640 supplemented with 10% FBS. The detached cells were collected and centrifuged at 1200 × *g* for 5 min. The supernatant was discarded, and the cell pellet was resuspended with 1 mL of 1× phosphate buffered saline (PBS) solution (Nacalai Tesque, Japan). A mixture of 10 µL cell suspension and 10 µL 0.4% (w/v) Trypan blue (Sigma-Aldrich, USA) was placed in a Neubauer chamber and viewed under light microscope. Cells that were stained blue were counted as nonviable, while clear cells were counted as viable cells. Cell concentration was calculated using the formula:

Cell concentration (cells/mL) = Average viable cell count × dilution factor × 10^4

Adherence assay

Adherence assay was carried out according to the method described by Othman *et al.* (2012), with slight modifications. On the day before the assay, Caco-2 cells were seeded into 24-well plates at a seeding density of 5 x 10^5 cells/mL and incubated overnight at 37 °C with 5% CO_2. Overnight cultures of *L. lactis* were also prepared in 10 mL GM17 broth. On the day of the assay, 100 µL of the overnight cultures were transferred into 10 mL fresh GM17 broth and cultured at 30 °C until OD_{600nm} reached approximately 1.0. Bacteria were centrifuged at 14,000 rpm for 1 min. Then, the supernatant was discarded, and the pellet was rinsed with 1× PBS. The pellet was resuspended in RPMI-1640 medium supplemented with 10% FBS and diluted to the desired colony forming unit per millilitre (CFU/mL), according to the multiplicity of infection (MOI) required, which were 0:1, 100:1, 250:1, 500:1, 750:1, and 1000:1 (bacteria:Caco-2 cells). The bacterial suspension was added to Caco-2 cells in 24-well plates and centrifuged for 5 min at 600 x *g*. Plates were incubated at 37 °C with 5% CO_2 for 2 h. After incubation, cells were washed twice with 1× PBS to remove bacteria that had not adhered to Caco-2 cells. Caco-2 cells were detached from the wells by incubating with 0.25% (w/v) Trypsin-EDTA for 5 min at 37 °C, followed by addition of RPMI-1640 medium supplemented with 10% FBS. The detached cells were collected into 15 mL tubes and the wells were washed with 1× PBS to collect the remaining cells. The cell suspension was centrifuged at 1,200 x *g* for 5 min. The supernatant was discarded, and cells were rinsed with 1× PBS to remove loosely adhering bacteria and excess trypsin. The pellet was resuspended in 1× PBS and an aliquot is taken for cell viability assessment as described previously. The remaining cell suspension was lysed with 100 µg/mL digitonin (Nacalai Tesque, Japan) for 30 min at 37 °C with 5% (v/v) CO_2. The cell suspension was serially-diluted in 1× PBS and plated on GM17 agar plates. Plates were incubated overnight at 30 °C and the number of colonies formed were recorded. The number of bacterial colonies formed on agar plates represents the total number of both adherent and internalised bacteria. To obtain the actual number of adhered bacteria, the number of bacteria CFU/mL obtained from adherence assay had to be subtracted with the number of bacteria CFU/mL obtained from internalisation assay. Bacterial adherence was expressed as the log CFU/mL of viable bacteria for every 5 × 10^5 cells/mL of Caco-2 cells, after 2 h of incubation at selected MOI.

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Internalisation assay

Internalisation assay was performed simultaneously as the adherence assay and using similar method, but with an additional step, as described by Othman *et al.* (2012). After 2 h incubation with bacteria, cells were washed with 1× PBS and incubated for 1 h with RPMI-1640 medium supplemented with 10% FBS containing 50 µg/mL gentamicin and 50 µg/mL kanamycin to eliminate non-internalised bacteria. The procedure for cell collection and plating were proceeded similarly as described for adherence assay. Bacterial internalisation was expressed as the log CFU/mL of viable bacteria for every 5 × 10^5 cells/mL of Caco-2 cells, after 2 h of incubation at selected MOI.

Data analysis

For both assays, three technical and three biological replicates were prepared. Bacterial adherence and internalisation data were presented as mean ± standard error mean, where n=3. Statistical analyses were performed using GraphPad Prism 9. Statistical significance of the mean values between groups was calculated using One-Way ANOVA test and considered significant at value of $p<0.05$.

RESULTS AND DISCUSSION

Assessment of multiplicity of infection (MOI) and Caco-2 cell viability

In order to determine the suitable MOI for interaction between *L. lactis* M4 and Caco-2 cells, adherence and internalisation assays were performed using various MOIs and the viability of Caco-2 cells were evaluated after the assays. The MOIs tested were 0:1, 100:1, 250:1, 500:1, 750:1, and 1000:1, which represented the ratio of bacteria to Caco-2 cells.

According to Figure 1, it is observed that there is a slight decrease in the percentage of viable Caco-2 cells with increasing MOI. However, even at the highest MOI of 1000:1, more than 70% of Caco-2 cells remained viable after 2 hours of incubation with *L. lactis* M4. It was also observed that the increase of MOI above 250:1 is followed by a gradual change in the colour of the cell culture media from pink to yellow and does not result in further increase in bacterial adherence and internalisation (data not included). The change in colour indicates a change in pH of the media containing phenol red, which turns yellow at pH below 6.4. Furthermore, according to one study, *L. lactis* NZ9000 internalisation was reported around log CFU/mL 2.5-3 at an MOI of 1000:1 (Yagnik *et al.*, 2016), which is slightly lower than the value observed in this study when an MOI of 250:1 was used (Figure 2). Based on these observations, it was concluded that *L. lactis* M4, in general, was not cytotoxic towards Caco-2 cells, and the change in pH and colour of culture media were most likely due to the bacteria competing with Caco-2 cells for nutrients and a result of lactic acid production by *L. lactis*. Therefore, to ensure a balance between Caco-2 cell viability and bacterial interaction, an MOI of 250:1 was selected as the suitable MOI for all *L. lactis* strains in subsequent assays. At this MOI, the calculated percentage of viable Caco-2 cells were 91.72% and 89.97% for adherence assay and internalisation assay, respectively.

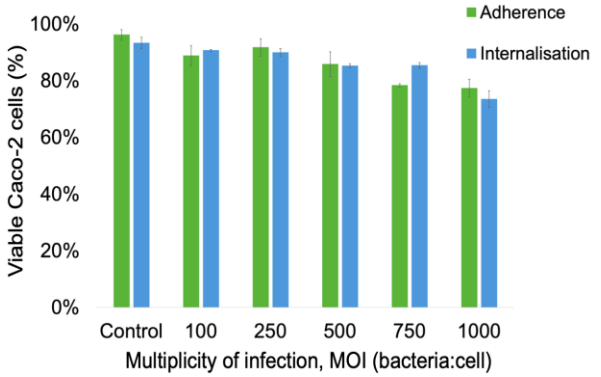

Figure 1: Percentage of viable Caco-2 cells after adherence and internalisation assays with *L. lactis* M4 at different MOIs. Data represents mean ± standard error mean (SEM), where n=3.

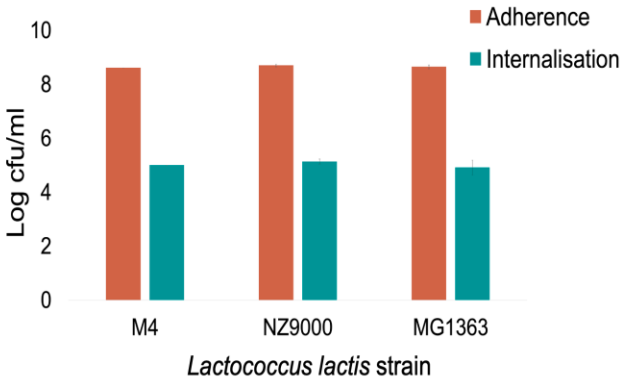

Figure 2: Adherence and internalisation of *Lactococcus lactis* strains with Caco-2 cells. Bacterial adherence and internalisation are expressed as log CFU/mL viable bacteria after 2 h of interaction at MOI of 250:1. Data represents mean ± standard error mean (SEM), where n = 3

Bacterial adherence and internalisation

Both bacterial adherence and internalisation are presented as log CFU/mL, as shown in Figure 2. Adherence of *L. lactis* M4 was recorded as log 8.61 CFU/mL compared to log 8.69 CFU/mL and log 8.65 CFU/mL adherence by *L. lactis* NZ9000 and MG1363, respectively. Bacterial internalisation was log 5.0 CFU/mL

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for *L. lactis* M4, log 5.13 CFU/mL for *L. lactis* NZ9000, and log 4.91 CFU/mL for *L. lactis* MG1363. Statistical analysis revealed that there were no significant differences between the adherence and internalisation of *L. lactis* M4 with the other two commercial strains.

Previous *in vitro* studies have recognised the probiotic potential of *L. lactis* based on its ability to adhere to intestinal epithelial cells such as Caco-2 and HT-29 cells (Kimoto *et al.*, 1999; Rahman *et al.*, 2014). Ohland and MacNaughton (2010) suggested that epithelial adherence by probiotic bacteria could be crucial for providing epithelial barrier function by competing with invading pathogens for binding sites that are present on epithelial cells and mucous layers. In addition to surface adherence, entry into target cell is also essential in order to achieve successful DNA delivery. The non-invasive and non-pathogenic nature of *L. lactis* is generally considered as a constraining factor for efficient DNA delivery. To overcome this, studies have been conducted to explore various strategies for improving bacterial internalisation. A very popular and widely discussed strategy is the development of recombinant invasive *L. lactis* strains expressing cell wall anchoring proteins or adhesion proteins from other known pathogenic bacteria. According to a study by Innocentin *et al.* (2009), recombinant *L. lactis* expressing either a *Listeria monocytogenes* internalin A or a *Staphylococcus aureus* fibronectin-binding protein A showed an increase in the internalisation rates in Caco-2 cells, compared to wild-type *L. lactis*. Despite that, these strategies have their own limitations whereby *L. lactis* expressing internalin A could not be tested using *in vivo* murine model because the internalin A that binds to human E-cadherin receptors are not able to do the same with murine receptors, while fibronectin-binding protein A requires a certain fibronectin concentration to be able to bind its receptors (De Azevedo *et al.*, 2012).

However, successful internalisation of *L. lactis* NZ9000 have been demonstrated, without requiring any modifications or additional treatments (Yagnik *et al.*, 2017). Therefore, this study was designed to focus solely on the natural adherent and internalisation capabilities of our locally-isolated *L. lactis* M4 strain, and no genetic modifications were involved. By avoiding the use of genetic modifications, this approach is expected to comply with the United States Food and Drug Administration (USFDA) requirements for future applications of this strain in human. Findings from this study are also coherent with another study, which also reported on the ability of this local strain to display bacterial interaction with human colorectal cancer cell line, SW620 cells (Faroque *et al.*, 2018). According to Faroque *et al.* (2018), *L. lactis* M4 showed significantly greater rate of bacterial adherence and internalisation in comparison to an established probiotic strain, *Lactobacillus reuteri* CI. 3.1.4. Despite that, no comparison was ever done with other commercial *L. lactis* strains and the investigation was only carried out using one cell line. Hence, in this study, the adherence and internalisation of *L. lactis* M4 were compared with commercial *L. lactis* strains NZ9000 and MG1363 because these two strains are commonly used in bactofection studies (Pontes *et al.*, 2011). Overall, our results showed that *L. lactis* M4 is indeed similar to commercial strains in terms of interaction abilities and could potentially be used in further studies involving the bacteria. Examples include using *L. lactis* for therapeutic protein expression studies and protein and DNA delivery studies.

CONCLUSION

In conclusion, the locally-isolated *L. lactis* strain M4 displays adherence and internalisation abilities, without showing signs of cytotoxicity towards Caco-2 cell. The rate of bacterial adherence and internalisation of this strain at MOI of 250:1 is comparable to commercially available *L. lactis* strains NZ9000 and MG1363. Thus, we concluded that this Malaysian strain has similar commercialisation properties as a potential DNA delivery vector and can be used in place of the existing commercial strains.

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