P-037 **Effect of microencapsulation on viability and cytokine production of *Lactobacillus salivarius* 29 in alginate/chitosan/alginate microcapsule**

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INTRODUCTION AND OBJECTIVES

Lactic acid bacteria (LAB) have been received tremendous scientific and commercial interest due to their enormous health benefits in the gastrointestinal tract. They can inhibit and reduce numbers of potentially harmful bacteria from the intestine (Gillian 2008). There are a bunch of literatures regarding the induction of pro- and anti-inflammatory cytokines consequently regulating host immunity by LAB *in vitro* and *in vivo* by activating macrophages (Chon 2009). Over production of pro-inflammatory cytokines such as TNF-α and IL-6 causes unbalanced auto-immunity leading to septic shock, inflammation and cytotoxicity. In contrast, anti-inflammatory cytokines inhibit the production of pro-inflammatory cytokines. Thus, balance between the pro- and anti-inflammatory cytokines production is important in host immune system.

Lactobacillus salivarius 29 (LS29), a novel LAB strain which is recently identified from pig fecal sample, can be used as a probiotic microorganism for therapeutic applications because it showed strong inhibition against *E. coli K88*, *E. coli O157*, *Salmonella enteritidis*, *Salmonella typhimurium* and some others (Islam 2011). To exert positive health benefits such as the production of cytokines, therapeutic live bacteria must be delivered to the intestine alive through oral administration. However, low oral bioavailability, poor survival and stability in acidic stomach and short residence time at the target site have made oral delivery of live bacteria as a most challenging and difficult field in the research arena.

Considering these-mentioned problems and importance, hence, we microencapsulated LS29 into alginate/chitosan/alginate (ACA) microcapsules for their efficient oral delivery. Encapsulation LS29 in ACA microcapsules improved their survivability in simulated gastric fluid (SGF, pH 2). Interestingly, higher percentage of LS29 survived in ACA microcapsules after freeze-drying compared to that without freeze-drying when stored at 4 °C. LS29 were released in simulated intestinal fluid (SIF, pH 7.2) from LS29-loaded microcapsules in a time-dependent manner. In addition, the ACA microencapsulated LS29 also revealed the immunomodulatory effect in macrophage.

MATERIALS AND METHODS

Encapsulation of LS29 into ACA microcapsules and freeze-drying

In brief, the mixture of sodium alginate with LS29 and 15% (v/v) glycerol as a cryoprotectant was dropped into 0.1M $CaCl_2$ by passing through cannula-like syringe in the presence of N_2 gas pressure. The final concentration of sodium alginate was 2 wt-%. The formed microcapsules were washed with 0.85% saline to remove unreacted $CaCl_2$. The microcapsules were then immersed in 0.8 wt: % chitosan solution for 30 min followed by two times washing. Chitosan-coated alginate microcapsules were finally coated with 0.1 wt-% of alginate for 10 min followed by washing. The prepared ACA microcapsules were stored at -75 °C for 6 h and freeze-dried for 18 h.

Survivability and release study

Milk-based media containing 12% non-fat skim milk, 2% glucose, 1% yeast extract and 0.05% cysteine were used to prepare SGF (pH 2) and SIF (pH 7.2). Survivability of free and encapsulated LS29 was determined by counting colony forming unit (CFU) after incubating in SGF at 37 °C with 100 RPM for 0, 10, 30, 60, 90 and 120 min. Similarly, survivability of free LS29 in SIF was determined. In addition, release of LS29 from LS29–loaded ACA microcapsules was studied.

Storage of LS29-loaded ACA microcapsules

Lyophilized LS2-loaded ACA microcapsules were stored at 4°C and room temperature (RT). Survivability of LS29 in LS29-loaded ACA microcapsules was monitored by CFU counting on every week till 8^{th} week.

Secretion of cytokines by LS29-loaded ACA microcapsules in macrophage

Mouse macrophage cell line RAW 264.7 were grown in 6-well plate at a cell density of 3×10^5 per well. The cells were treated with free LS29 as well as LS29-loaded ACA microcapsules (1×10^6 CFU/ml), unloaded ACA microcapsules. LPS (1 μg/ml) and only culture media were used as a positive and negative control, respectively. Cytokines (TNF-α and IL-10) produced by RAW264.7 were quantified by ELISA kit as per manufacturer's protocol.

RESULTS AND DISCUSSION

The ACA microcapsules were successfully prepared and characterized for their efficacy for oral delivery of LS29.

Survivability of free LS29 rapidly reduced to zero, when incubated in SGF at 37 °C and 100 RPM for 120 min (data not shown). The survival of free LS29 was decreased by 40% after 60 min and 60% after 90 min in SGF at 37 °C and 100 RPM. No viable bacteria were detected in SGF at 120 min. In contrast, 100% of LS29 continued their viability in SIF even after 120 min.

In order to protect LS29 from harsh gastric condition, LS29 were encapsulated into ACA microcapsules and also freeze-dried. About 99% of LS29 were encapsulated into ACA microcapsules. However, freeze-drying reduced the encapsulated LS29 to 77%. LS29-loaded ACA (ACA-LS29) and freeze-dried LS29-loaded ACA (FDACA-LS29) microcapsules were incubated in SGF (pH 2) at 37 °C and 100 RPM for 120 min. About 70% of LS29 were survived after 120 min by both ACA-LS29 and FDACA-LS29 microcapsules (Figure 1).

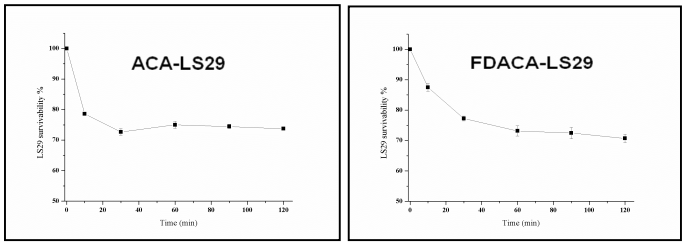

Figure 1. Survivability of ACA microencapsulated LS29 before and after freeze-drying at pH 2.

About 88% of LS29 were released within 0.5 h from ACA-LS29 microcapsules. In contrast, only 65% of LS29 were released from FDACA-LS29 microcapsules and 97% were released after 7 h. The result implies that freeze-drying can extend the release of LS29 from ACA microcapsules (data not shown).

The survival of LS29 as free and encapsulated form (in ACA and FDACA microcapsule) was investigated at 4 °C and room temperature (RT) for 8 weeks. LS29 stored at 4 °C and RT could not survive more than 2 weeks and 6 weeks, respectively (data not shown). Survivability of LS29 in ACA was reduced to 65% after 2 weeks when stored at 4°C. Interestingly, FDACA capsules increased the survivability of LS29 more than 70% till 7 week of storage at 4 °C (Figure 2).

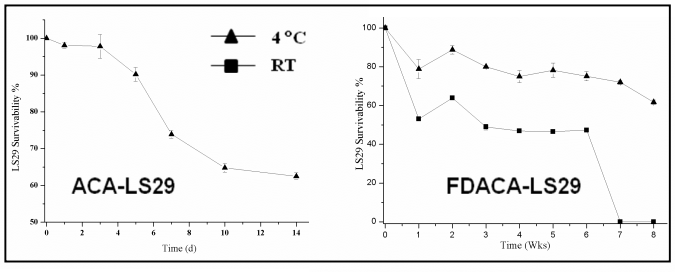

Figure 2. Survival of ACA microencapsulated LS29 before and after freeze-drying at different storage condition.

In order to observe the immunological modulation of LS29 before and after encapsulation, most popularly used mouse macrophage cells, RAW264.7 were stimulated with unloaded ACA microcapsules, LPS, free LS29 and LS29-loaded ACA microcapsules for 24 h. Morphological changes of RAW264.7 cells were observed after treatment with LPS (2 μg), free LS29 and LS29-loaded ACA microcapsules, indicating the activation of the cells. No morphological changes were observed under microscope in control (without any treatment) and ACA treated cells (data not shown). After 24 h treatment, TNF-α and IL-10 concentration in supernatant was detected by ELISA (Figure 3). The result showed significant induction of TNF-α by LS29 treatment followed by LPS and LS29-loaded ACA microcapsules. Similarly, LPS, LS29 and LS29-loaded ACA microcapsules induced the production of IL-10. Interestingly, cytokines production is lower by encapsulated LS29 than free LS29 treatment. This may be due to the controlled release of LS29 from LS29-loaded ACA microcapsules. Induction of TNF-α and IL-10 implies that LS29 can immunomodulate without leading to imbalance the immune system in the host.

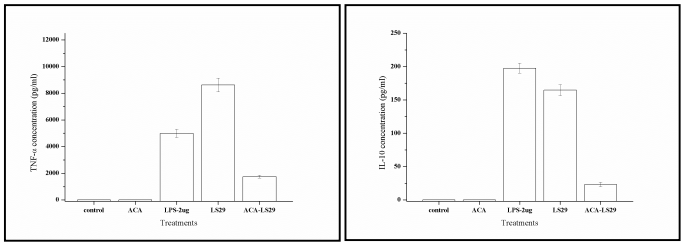

Figure 3. Secretion of TNF-α and IL-10 in Raw264.7.

CONCLUSIONS

We have successfully prepared LS29-loaded ACA microcapsules and found improved survivability of the loaded LS29 in gastric condition (pH 2) even after freeze-drying of the microcapsules. Interestingly, control release of LS29 in SIF and survivability of LS29 during storage were found to be improved in freeze-dried ACA microcapsules. Importantly, the immunomodulatory effect of LS29 was observed before and after encapsulation *in vitro*, suggesting potentials of this encapsulation system loaded with bioactive agents such as LS29 to modulate immune system of the host.

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