Odun-Ayo et al., Afr J Tradit Complement Altern Med. (2016) 13(2):101-109
http://dx.doi.org/10.4314/ajtcam.v13i2.13

IMPROVING THE SURVIVAL OF PROBIOTIC IN SIMULATED CONDITIONS AND AZOXYMETHANE-INDUCED COLON TUMOUR BEARING MICE USING MODIFIED CITRUS PECTIN-ALGINATE MICROENCAPSULATION

[1]Frederick Odun-Ayo, [1*]John Mellem and [2]Lalini Reddy

[1]Department of Biotechnology and Food Technology, Durban University of Technology, Steve Biko, KwaZulu-Natal, South Africa
[2]Faculty of Applied Science, Cape Peninsula University of Technology, Cape Town, South Africa
***Corresponding author: E-mail:** johnm@dut.ac.za

Abstract

Background: For a probiotic to be viable it needs to be preserved at a recommended minimum level of 6–7 $\log_{10}$cfu/g in the product being consumed, as suggested by the International Dairy Federation. Different biopolymer matrices have been used for encapsulation of probiotic; however, loss of viability is still a challenge.
Materials and Methods: Modified citrus pectin-alginate microbeads containing *Lactobacillus acidophilus* ATCC 4356 was developed. Efficiency of the microbeads was evaluated in simulated conditions of the gastrointestinal tract and in Balb/c mice induced with colon tumor. Genomic identification of faecal lactobacilli samples from treated mice was also performed.
Results: The Modified citrus pectin-alginate probiotic microbeads significantly enhanced the viability of *Lactobacillus acidophilus* ATCC 4356 compared to the control ($p< 0.05$) both *in vitro* and *in vivo*. Exposure of the modified citrus pectin-alginate microbeads to 3 hours of simulated gastric juice resulted in 82.7% survival of *L. acidophilus* ATCC 4356. Also, the number of faecal lactobacilli in the modified citrus pectin-alginate probiotic treated mice increased by 10.2% after 28 days.
Conclusion: Modified citrus pectin-alginate is a novel effective means of oral delivery of bacterial cells and bioactive compounds. Modified citrus pectin-alginate can be used in probiotic therapy which may improve the prevention of colon cancer.

Key words: Modified citrus pectin, alginate, probiotic, and microencapsulation.

Introduction

The use of pro-biotic microorganisms in the food industry and complementary medicine has gained great interest (Gbassi et al., 2011; Mitropoulou et al., 2013). Probiotic microorganisms are live supplement incorporated sometimes into functional foods which gives health benefit to host when consumed. These microorganisms when consumed pass through the gastrointestinal tract (GIT) during which viability may be lost before they reach their target site. Therefore, the viability of probiotic microorganisms during storage and transit in the GIT is of paramount importance (Anal and Singh, 2007; Gebara et al., 2013). Probiotics are defined as "live microorganisms which when administered orally in adequate amount confer a health benefit on the host" (FAO, 2002). Attention has been focused on decreasing the risk of cancer, particularly through the consumption of probiotics and increase in dietary fibre intake (Mandal et al., 2006). However, the colonic flora is able to produce substances with toxic or tumour-promoting activities when metabolising some dietary compounds in animal model. Therefore, the alteration/manipulation of intestinal bacterial composition through consumption of probiotics and composition of the diet may have the potential to reduce the risk of colon cancer by stimulating the immune system, regulating inflammations in the gut, decreasing incidence of infections and binding toxic substances (Capurso et al., 2006). A clinical trial was conducted where colonic microflora, *Lactobacillus acidophilus* showed anti-carcinogenic activity in humans (Hansen et al., 2002). Also, studies have demonstrated that the inclusion of probiotics in diet reduces the risk of cancer (Chen and Chen, 2007). O'Keefe et al. (2007) reported that a low endogenous faecal lactobacilli count in the colonic mucosal biopsies leads to higher incidence of colon cancer in African Americans.

Encapsulation successfully protects probiotic bacterial cells against adverse environmental and intestinal effect, thereby releasing them in their viable and metabolic active states in the intestine at under specified pH conditions. Various materials have been employed in probiotic encapsulation, but fast and easy inflow of water and other liquids through some of the matrices is a limitation (Anal and Singh, 2007). Extensive studies found alginate suitable for probiotic encapsualtion because of its lack of toxicity, ability to entrap living microorganisms and it is generally regarded as safe "GRAS" (Dinakar and Mistry, 1994; Gombotz and Wee, 2012). Modified pectin (MP) is a complex water soluble indigestible polysaccharide used as a dietary supplement to promote cell growth. MP, rich in β-galactose, is potentially safe, non-toxic and it possesses a unique bioactivity of inhibiting carcinogenesis (Morris, 2009; Maxwell et al., 2012).

Azoxymethane (AOM), a metabolite of 1, 2- dimethylhydrazine, is a potent specific carcinogen used to induce colon cancer in mice and rats (Bissahoyo et al., 2005; Tanaka, 2009). The AOM mouse model has been extensively used in the study of underlying mechanism of sporadic colon cancer in humans. The response of AOM-induced colorectal cancer mouse model mimics the occurrence of non-familial colon tumour particularly sporadic colon cancer in humans (Chen and Huang, 2009). Although few animal studies have shown that probiotics alone or synbiotics (probiotic and prebiotic) can reduce the incidence of precancerous lesions, but the mechanism by which this health benefit comes into play remains unclear and may be dynamic in nature (Capurso et al., 2006). The potential role of modified pectin (MP) and probiotics in the prevention of carcinogenesis has prompted the need to understand their synergistic influence on colon microflora. Therefore, the aim of this study was to investigate the effect of modified citrus pectin-alginate on the survival of encapsulated *Lactobacillus acidophilus* ATTC 4356 *in vitro* (gastric and intestinal juice) and *in vivo* (Balb/c mouse model of AOM-induced colon tumour).

http://dx.doi.org/10.4314/ajtcam.v13i2.13

Materials and Methods

Plant Material

Modified citrus pectin [(MCP); ecoNugenics Inc. (CA, USA), extracted from the peel pith of orange fruit, *Citrus sp*.]. This was obtained in form of a fine powder and stored at room temperature. Alginate sodium [Sigma Aldrich (St. Louis, M.O. USA), extracted from the cell wall of brown seaweed mostly found in cold water regions] was obtained in powder form.

Preparation of Modified Citrus Pectin-Alginate Lactobacillus Acidophilus ATCC 4356 Micro-beads

Microencapsulation of the pro-biotic was performed aseptically at room temperature. Frozen stock culture of *L. acidophilus* ATCC 4356 was rehydrated and grown in MRS agar/broth (Sigma Aldrich, St. Louis, M.O. USA) at 37°C for 48 hours under aerobic and anaerobic condition using anaerocult®. Fresh cell suspensions of about 9-10 $\log_{10}$cfu/g were prepared for each microencapsulation procedure. Both the modified citrus pectin alginate (MCPA) and alginate calcium (AP) microbeads were produced separately using modified emulsification method (Homayouni et al., 2008). Modified citrus pectin (8.5%) and sodium alginate (2%) polymers, incorporated with hi-maize resistant starch (2%) (National Starch Food Innovation, Wadeville, Guateng) were agitated in distilled water for 10 min to produce the MCPA microbeads. For the AP microbeads, sodium alginate (2%) and hi-maize resistant starch (2%) were used. Cell suspension (1 ml) of *L. acidophilus* ATCC 4356 was added to the polymer mixtures in 300 ml of canola oil. The mixture was emulsified by adding lecithin (0.1%) with a constant agitation at 1400 rpm for 40 min. Calcium chloride (0.1 M) solution was added to the polymer mixtures to harden the beads and agitated for 5 min. Thereafter, the MCPA or AP probiotic microbeads were collected by centrifugation at 4000 rpm for 5 min at 4°C. The microbeads of *L. acidophilus* ATCC 4356 were immersed in 100 ml of chitosan solution (Sigma Aldrich, St. Louis, M.O, USA) and agitated at 1400 rpm for 15 min on magnetic stirrer for coating. The microbeads were retrieved, washed with saline solution (0.9% NaCl) and stored in sodium glycerol (0.9% NaCl, 5% glycerol) solution at 4°C. Free cells of *L. acidophilus* ATCC 4356 were stored in saline solution at 4°C

The morphology and particle size (µm) of microbeads chosen at random were measured under MOTIC optical microscope (Motic images Plus 2.0 software, Hong Kong, Asia) and images captured with camera (Moticam 2500, Hong Kong, Asia). The size of each microbead is presented as mean ± standard deviation (SD).

In Vitro:
Enumeration of Lactobacillus Acidophilus ATCC 4356

The viability of the encapsulated *L. acidophilus* ATCC 4356 in MCPA and AP microbeads were determined by vigorously homogenizing 1 g of the microbeads in 9 ml of sterile phosphate buffer solution (PBS) pH (7.4) for 10 min (Sheu and Marshall, 1993; Annan et al., 2008). Cell suspensions (100 µl) were plated on MRS agar and incubated for 48 hours. Viable cells growth recorded in log10 cfu/ g were enumerated using automated colony Doc-It® imaging station (UVP, C.A, USA). However, the microencapsulation yield (EY) which is a combined measurement of the efficacy of encapsulation and survival of viable cells during the microencapsulation procedure was calculated using the formula:

$$EY = (N / N_0) \text{ X } 100$$

Where N is the number of viable encapsulated cells released from the microbeads ($\log_{10}$cfu/ g) and N_0 is the number of free cells added to the biopolymer matrix emulsion.

Determination of Survival of Free and Microencapsulated Lactobacillus Acidophilus ATCC 4356 in Simulated Gastric and Intestinal Juice

Simulated gastric juice (SGJ) was prepared as follows: 9 g/l of NaCl and 3 g/l of pepsin (St. Louis, M.O. USA) with final pH 2.0 by 0.1 M hydrochloric acid (HCl). Simulated intestinal juice (SIJ) was prepared as follows: 3% (w/v) bile salt (Sigma Aldrich, St. Louis, M.O. USA), 6.5 g/l NaCl, 0.835 g/l KCl, 0.22 g/l, $CaCl_2$ and 1.386 g/l $NaHCO_3$ with final pH 8.0 by 0.1 M NaOH. Then, 1 g of the MCPA and AP probiotic microbeads was homogenized in 9 ml of SGJ and SIJ and incubated at 37°C for 30 min, 60 min, 120 min and 180 min with constant agitation at 60 rpm. At each specified time interval, microbeads were washed with saline solution, diluted serially and enumeration of *L. acidophilus* ATCC 4356 cells was done as described above. All microbeads samples were treated in triplicates.

In vivo:
Animal Model

This experiment was carried out at the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN) in accordance with approved standard protocols for animal treatment and with post institutional ethics approval (063/13/Animal and 084/14/Animal). Seven week old male Balb/c mice weighing 20 – 25 g were bred in-house under a controlled condition of humidity (50 ± 10%) and temperature (23 ± 2°C) on a 12 hours light/12 hours dark cycle. Mice were allowed free access to water and food. During the experiment, mice were carefully observed for any toxic effect, unusual behaviour and rectal bleeding. In addition, body weight for each mouse were recorded and monitored weekly.

Azoxymethane (AOM) Treatment

Twenty-five micro-litre of AOM (13.4 Molarity, ≥98%, Sigma-Aldrich Co., St. Louis, USA) was reconstituted in 500 µl sterile phosphate-buffered saline (PBS) to prepare a working concentration of 1 mg/ ml AOM. The administration of AOM dose to each mouse was dependent on the body weight of the mouse**.** Each mouse was pre-treated with 15 mg/kg AOM intraperitoneally once a week for four consecutive weeks. The AOM dose and time response was optimised prior to the start of the animal study.

http://dx.doi.org/10.4314/ajtcam.v13i2.13

Modified Citrus Pectin-Alginate and Alginate Calcium Probiotic Micro-beads Treatment

The total study population (n=40) consisted of 4 groups (n=10 each). Each group of mice was orally administered 0.2 ml MCPA- and AP probiotic microbeads, MCP and water (control) for 28 days. Faecal samples were obtained for each group of mice at the initial day (day 0) prior the probiotic microbeads treatments and days 7, 14 and 28.

Microbiological Analysis of Faecal Bacteria

The faecal samples were transported in an ice pack bag and processed within 12 hours of collection. Samples were serially diluted in saline (0.9% NaCl) from 10^1 up to 10^{10} and inoculated on MRS agar for 48 hours at 37°C. Colonies were selected randomly from each faecal sample plates count of 30 – 300 cfu/ml. Identification of isolates from pure cultures was done based on the following parameters: colony morphology, gram stain and cell morphology.

Genomic DNA Sequence Analysis of Faecal Bacteria

DNA of bacterial isolates was extracted with Zymo spin™ IIC (Zymo Research Corporation, USA). PCR amplification of the DNA sample fragments of the 16s region was obtained using DreamTaq (Thermo scientific Fermentas) and primers, 27F: 5´ GAGTTTGATCCTGGCTCAG and 1492R: 5´ GGTTACCTTGTTACGACT 3´. A total volume of 50ul containing 25ul of DreamTaq Green PCR master mix (DreamTaq DNA polymerase, optimised DreamTaq green buffer, $MgCl_2$ and dNTPs), 1.0 µM of each primers, 1 µg of Template DNA and water, nuclease-free of PCR reaction. PCR was started by initial denaturation of template DNA at 95°C for 3 min at 1 cycle then second denaturation at 95°C for 30 s followed by primer annealing 25 cycles for 30 s, extension at 72°C and final extension for 15 min at the same temperature. Amplicons were separated on 1% agarose Gel followed by staining with GRGReen. Exo/SAP amplicon purification was used directly on the PCR fragments. The ultra-pure DNA fragment was loaded into the ABI 3500 XL sequence analyzer. Consensus sequence and blast algorithm were performed in CLC Bio (Inqaba biotech, SA) and compared with the known ones available in the Genbank database.

Data Analysis

Data were subjected to two way analysis variance (ANOVA) and Tukey's test to determine the significant differences among the means of microbeads using Graphpad Prism software SSPS version 17.0 for Windows (SSPS, Chicago, Illinois, USA). Results were presented as means ± standard deviation (SD) and statistical significance was set as ($p < 0.05$).

Results and Discussion

In vitro:

Size and Encapsulation Yield of Lactobacillus Acidophilus ATCC 4356 in Modified Citrus Pectin-Alginate and Alginate Pectin Micro-beads

In this study, the average mean diameter of the MCPA microbeads ranged from 220.89 ± 2.6 µm to 685.19 ± 0.7 µm which was significantly different from the AP microbeads which ranged from 147.61 ± 2.6 µm to 258.09 ± 2.6 µm ($p < 0.05$). The high concentration of modified citrus pectin blended with alginate calcium could be responsible for the large size of the MCPA microbeads. Chávarri et al. (2010) and Shi et al. (2013) found that polymer matrix with highest concentration produces large microsphere. Similarly, Sandoval-Castilla et al. (2010) found alginate (0.5%) without amidated pectin produced 710 µm microbeads size. When the concentration of amidated pectin was increased from 2 – 3%, the size of the bead also increased simultaneously from 930 – 970 µm. However, the particle size of the microbead may depend on the encapsulation technique involved. Emulsification technique mostly produces small diameter (25 µm – 2 mm) compared to extrusion which produces a large size (2 – 5 mm). The average viable count of *L. acidophilus* ATCC 4356 in the MCPA microbeads was found to be 8.16 ± 0.06 $\log_{10}$cfu/g while it was 8.10 ± 0.04 $\log_{10}$cfu/g in the AP microbeads. Chávarri et al. (2010) demonstrated that some strains of probiotic bacteria may be sensitive to the polymer material used for encapsulation which may lead to a low encapsulation yield. The entrapment of *Lactobacillus gasseri* and *Bifidobacterium bifidum* in alginate and quercetin reduces the encapsulation yield ranging from 19.5 – 22.2%. In our study, both the MCPA and AP microbeads showed a high microencapsulation yield of 88.6 ± 0.8% and 88.0 ± 0.4% respectively ($p > 0.05$). Sandoval-Castilla et al. (2010) found that alginate + pectin capsules formed a significantly higher entrapment efficiency compared to alginate alone which corroborates our findings. In the literature, alginate calcium microbeads are more spherical in shape unlike the alginate pectin calcium microbeads that are either vermiform appendix or less spherical (Pillay and Fassihi, 1999; Díaz-Rojas et al., 2004; Sandoval-Castilla et al., 2010; Shi et al., 2013). In this study, the MCPA microbeads were more of spherical than vermiform appendix (Figure 1). This result could be attributed to the coating effect by chitosan. The availability of carboxyl ions may increase the adsorption of chitosan to the modified pectin-alginate calcium particle surface thereby forming a spherical shape.

http://dx.doi.org/10.4314/ajtcam.v13i2.13

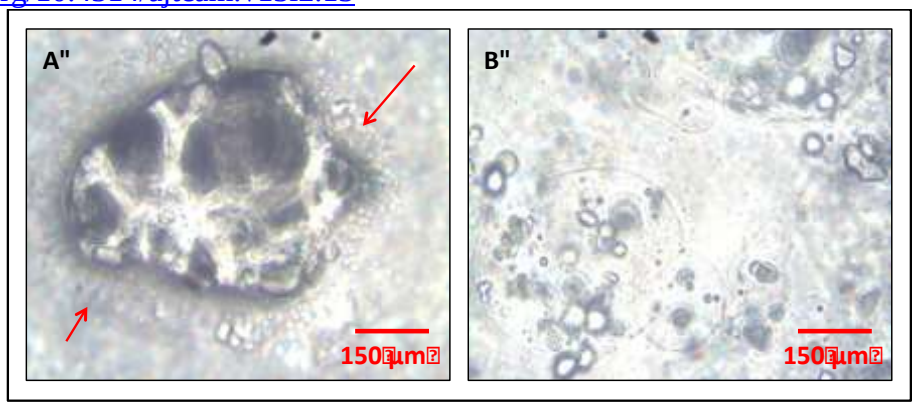

Figure 1: The morphology of A - MCPA (modified citrus pectin alginate probiotic microbead) and B - AP (alginate calcium probiotic microbead) particles produced by modified emulsification with arrows indicating the chitosan coating effect.

Stability of Free and Microencapsulated Lactobacillus Acidophilus ATCC 4356 in Storage

Stability of the microencapsulated probiotics at 4°C for 28 days was examined. Microencapsulated *L. acidophilus* ATCC 4356 had a significantly higher survival rate when compared to the free cell ($p < 0.05$) (Figure 2). The viability of *L. acidophilus* ATCC 4356 in the MCPA and AP microbeads were reduced by 1.13 $\log_{10}$cfu/g (13.5%) and 1.38 $\log_{10}$cfu/g (17.2%) respectively while the free *L. acidophilus* ATCC 4356 was significantly reduced by 6.75 $\log_{10}$cfu/g (70.4%) ($p < 0.05$). Similarly, Brinques and Ayub (2011) found the highest viability of *Lactobacillus plantarum* in a mixture of 2% sodium alginate + 2% pectin while the lowest viability was found in 4% pectin. The significant survival of *L. acidophilus* ATCC 4356 in the MCPA microbeads at 4°C was attributed to the prebiotic effect of hi-maize resistant starch incorporated in the blend of modified citrus pectin and alginate calcium microbeads. Also, the cryogenic effect of the sodium glycerol during storage of the microbeads at 4°C may give cryo protection to the microencapsulated *L. acidophilus* ATCC 4356 compared to the free *L. acidophilus* ATCC 4356. The viability of *L. acidophilus* ATCC 4356 was 86.5% in the MCPA microbeads which is similar to Sheu and Marshall (1993). From our study, the blend of modified citrus pectin + alginate-calcium coated with chitosan in glycerol is an improved mixture of polymers which enhanced the stability and survival of *L. acidophilus* ATCC 4356 under refrigerated condition.

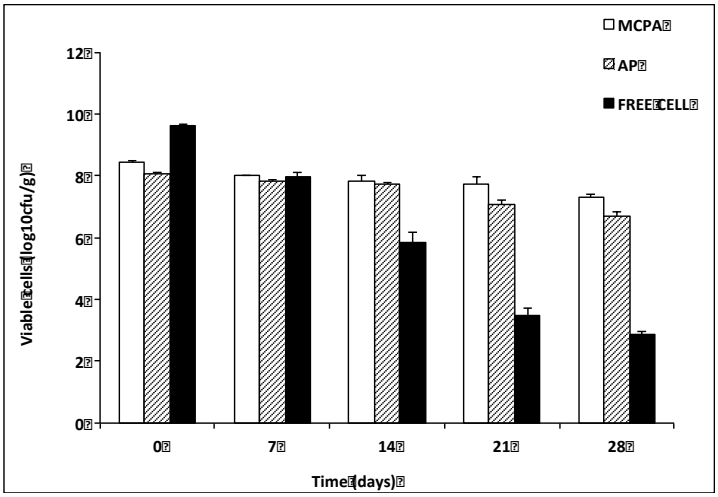

Figure 2: The viability of *L. acidophilus ATCC 4356* as free and encapsulated cells during storage at 4°C. MCPA - modified citrus pectin alginate probiotic microbeads, AP - alginate calcium probiotic microbeads and free cell – *L. acidophilus* ATCC 4356 cells. Each column represents mean ± standard deviation (n=3).

Survival of Free and Microencapsulated Lactobacillus Acidophilus ATCC 4356 in Simulated Gastric Juice

The sensitivity of free probiotic bacteria to low pH (1.5 – 3.0) in the stomach reduces their survival considerably. Thus, the testing of the probiotic microbeads in simulated physiological conditions (gastric) was performed. At a pH 1.2, no viable free *L. acidophilus* ATCC 4356 was found after 30 min in SGJ while the number of encapsulated *L. acidophilus* ATCC 4356 cells in both MCPA and AP microbeads was below the detection limit, that is less than 2 $\log_{10}$cfu/g) after 1 hour which is similar to the data reported by Gebara et al. (2013). Also, Mandal et al. (2006) and Ortakci and Sert (2012) observed a drastic decrease in the number of free probiotic cells when exposed to SGJ (pH 1.5) for 30 min. At pH 2.0, we observed that the number of free *L. acidophilus* ATCC 4356 in SGJ reduced significantly by 4.01 $\log_{10}$cfu/g (43.6%) compared to 0.55 $\log_{10}$cfu/g (5.9%) and 0.91 $\log_{10}$cfu/g (10.3%) in the encapsulated *L. acidophilus* ATCC 4356 MCPA and AP microbeads respectively after 180 min in SGJ ($p < 0.05$) (Figure 3). The viable number of *L. acidophilus* ATCC 4356 in these polymer matrices (MCPA and AP) was above 10^7 that is > 7 $\log_{10}$cfu/g which is the required minimum concentration of probiotic therapy able to confer health benefit (Anal and Singh, 2007). Ortakci and Sert (2012) found that *L. acidophilus* ATCC 4356 count in alginate calcium microbeads was reduced by 0.25 $\log_{10}$cfu/g after 30 min in SGJ. A little more than 1 $\log_{10}$cfu/g of *L. gasseri* and less than 1 $\log_{10}$cfu/g of *Bifidobacterium bifidum* in alginate-chitosan capsules were lost after 120 min in artificial gastric juice (Chávarri et al. (2010).

http://dx.doi.org/10.4314/ajtcam.v13i2.13

The improved survival of *L. acidophilus* ATCC 4356 in the MCPA micro-beads was attributed to the resilient and cohesive cross-linking network between modified citrus pectin and alginate calcium polymers. In the AP microbeads, calcium and chitosan polycations binds competitively to the carboxyl anions in alginate molecules. In the MCPA microbeads, the abundance of carboxyl polyanions (MCP and alginate) equivalently binds to calcium and chitosan polycations which forms a strong polyelectrolyte complex. This synergistic effect produced a strong trapping matrix which reduced the porosity of the bead wall.

Viability of Free and Microencapsulated *Lactobacillus Acidophilus* ATCC 4356 in Simulated Intestinal Juice

This is to determine the stability of both MCPA and AP pro-biotic microbeads in the presence of bile salt in SIJ (pH 8.0) (Figure 3). In this study, free *L. acidophilus* ATCC 4356 cells were found not to survive in SIJ after 30 min. After 60 min, the number of cell in the AP microbeads decreased from 8.10 ± 0.04 $\log_{10}$cfu/g to 3.35 $\log_{10}$cfu/g and further reduced to 2 $\log_{10}$cfu/g (66.3%) at 120 min. In the MCPA microbeads, *L. acidophilus* ATCC 4356 reduced from 8.16 ± 0.06 $\log_{10}$cfu/g to 4.53 ± 0.10 $\log_{10}$cfu/g after 180 min in SIJ. The viability of *L. acidophilus* ATCC 4356 cells in the MCPA was significant compared to the AP microbeads ($p < 0.05$). Similar to our findings, Shi et al. (2013) observed the total loss of free *L. bulgaricus* after 1 h exposure to bile salt solution. Trindade and Grosso (2000) found that alginate calcium beads did not protect *B. bifidum* and *L. acidophilus* from 2% and 3% bile salt action. On the contrary, Ortakci and Sert (2012) found no reduction in the number of both free and encapsulated *L. acidophilus* ATCC 4356 in 1.2% bile juice. Studies demonstrated that some probiotic strains are unaffected by intestinal bile action (Ortakci et al., 2012; Ortakci and Sert, 2012) while some are susceptible (Clark and Martin, 1994; Hansen et al., 2002; Chávarri et al., 2010; Shi et al., 2013; Trabelsi et al., 2013). Also, a high concentration of bile salt may disintegrate the bacterial cell wall integrity. In this regard, the outcomes of different studies varied as a result of different concentrations of bile salt solutions (0.3 – 4%) and pH (6 – 8). Figures 4 and 5 show the morphology of the MCPA and AP microbead particles containing *L. acidophilus* ATCC 4356 in SGJ and SIJ respectively after sequential exposure for 3 hours.

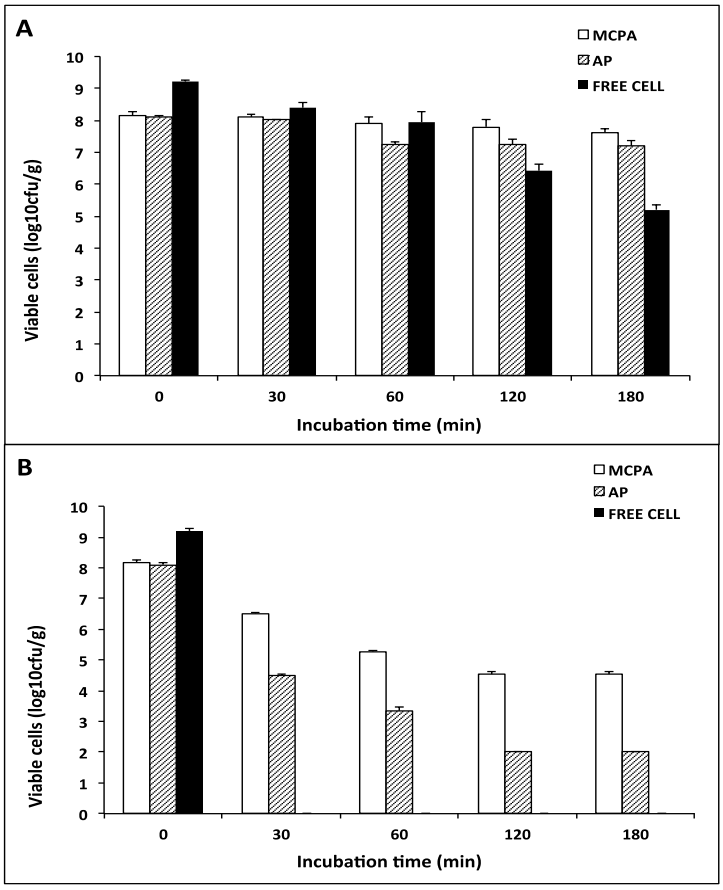

Figure 3: The viability of *L. acidophilus* ATCC 4356 as free and encapsulated cells during exposure to A - simulated gastric juice (SGJ) at pH 2 and B - simulated intestinal juice (SIJ) at pH 8 for 30, 60, 120 and 180 min. Each column represents mean ± standard deviation (n=3). [MCPA - modified citrus pectin alginate probiotic microbeads, A - alginate calcium probiotic microbeads, free cell – *L. acidophilus* ATCC 4356 cells]

http://dx.doi.org/10.4314/ajtcam.v13i2.13

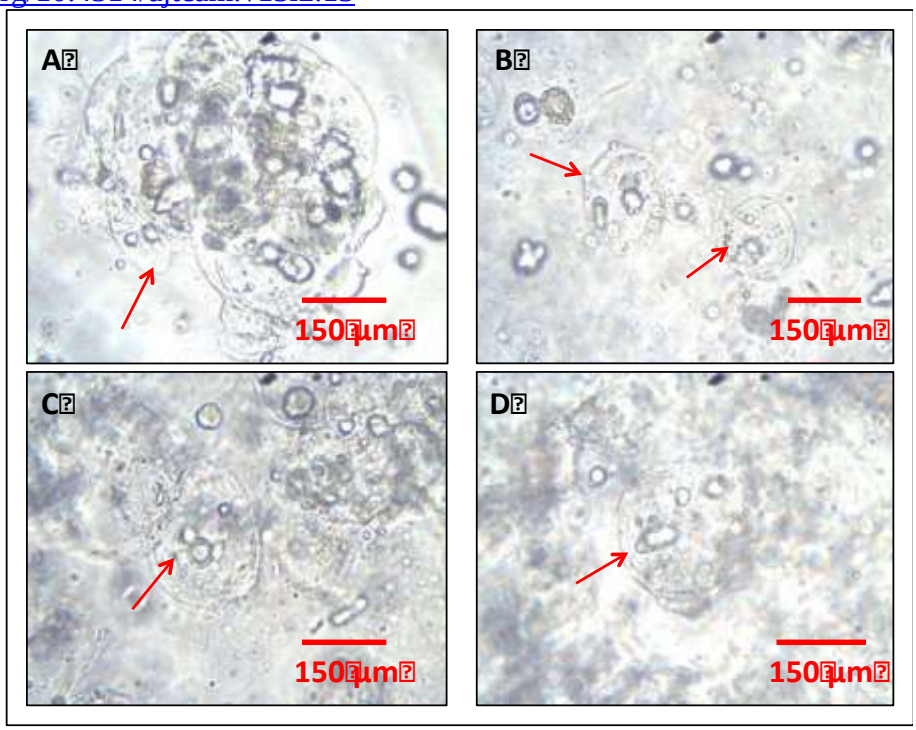

Figure 4: The morphology of MCPA (modified citrus pectin alginate) microbead particles containing *L. acidophilus* ATCC 4356 after (A) 30 min (B) 60 min (C) 120 min and (D) 180 min exposure to simulated gastric juice (SGJ) at pH 2. Microbead particles encapsulating *L. acidophilus* ATCC 4356 indicated by the arrows.

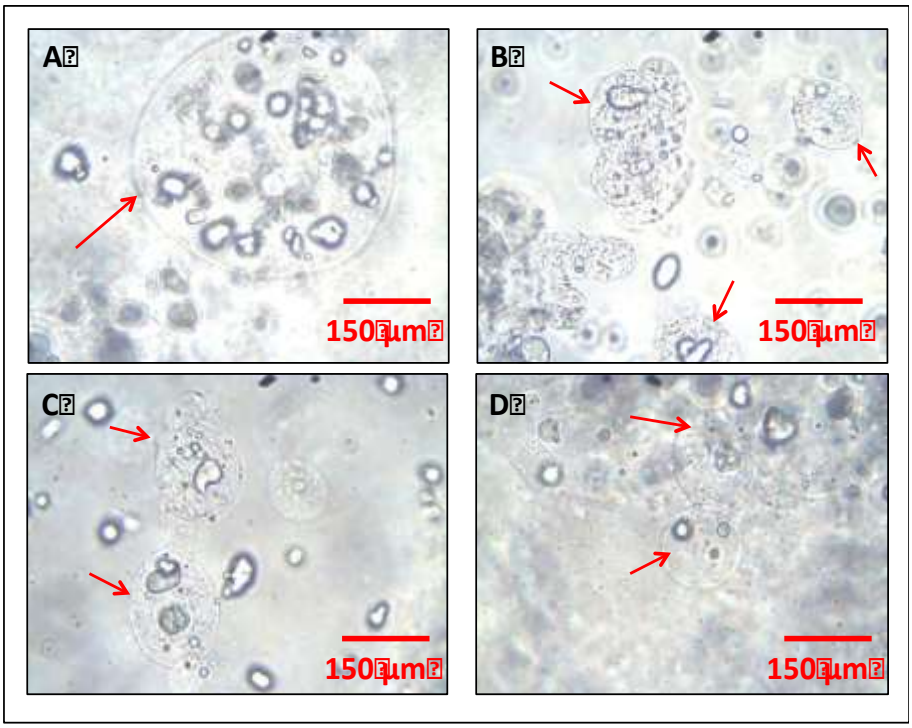

Figure 5: The morphology of AP (alginate probiotic) microbead particles containing *L. acidophilus* ATCC 4356 after (A) 30 min (B) 60 min (C) 120 min and (D) 180 min exposure to simulated intestinal juice (SIJ) at pH 8. Microbead particles encapsulating *L. acidophilus* ATCC 4356 is indicated by the arrows.

In vivo:
Quantification and Analysis of Faecal Bacteria

At day 7 of treatment, the amount of faecal lactobacilli in the MCPA probiotic treated group was increased by 0.38 ± 0.12 $\log_{10}$cfu/g (5%) while in both the AP and MCP treated groups, 0.02 ± 0.05 $\log_{10}$cfu/g (0.2%) increase was observed ($p > 0.05$). At day 14, the change in amount of the faecal lactobacilli in the MCPA probiotic treated group increased significantly by 0.75 ± 0.4 $\log_{10}$cfu/g (9.6%) compared to 0.32 ± 0.04 $\log_{10}$cfu/g (4%) and 0.01 ± 0.03 $\log_{10}$cfu/g (0.4%) in the AP probiotic and MCP treated groups respectively ($p < 0.05$). At day 28 of treatment, the faecal lactobacilli count in the MCPA- and AP probiotic treated groups further increased by 10.2% and 6% respectively. In the MCP treated group, the number of faecal lactobacilli reduced by 0.6% below

http://dx.doi.org/10.4314/ajtcam.v13i2.13

the initial baseline level (before treatment), although not statistically significant ($p > 0.05$). The amount of lactobacilli in the control group was also reduced by 0.1 ± 0.4 $\log_{10}$cfu/g (1.2%) ($p > 0.05$). The difference in number of lactobacilli before and after probiotic consumption was significantly increased only in the MCPA probiotic treated group compared to the AP probiotic, MCP treated and control groups ($p < 0.01$) (Figure 6). The changes in the number of faecal microflora observed in the treated mice supports the observation made by Mountzouris et al. (2006) that the colon microflora responds dynamically to change in dietary intake. MCP failed to increase the number of faecal lactobacilli in the MCP treated mice which corroborates with data reported by Biagi et al. (2010). However, the disintegration of chitosan coating and fermentation of the modified citrus pectin and resistant starch by *L. acidophilus* ATCC 4356 and intestinal microflora leads to the increased production of short chain fatty acids (SFCAs), gases and butyrate. Butyrate is the preferred energy source for colonic epithelial cells and growth of the faecal lactobacilli. These SFCAs have the ability to stimulate bile salt hydrolase which leads to the deconjugation of bile acid thereby reducing the effect of bile in the intestine (Ooi and Liong, 2010).

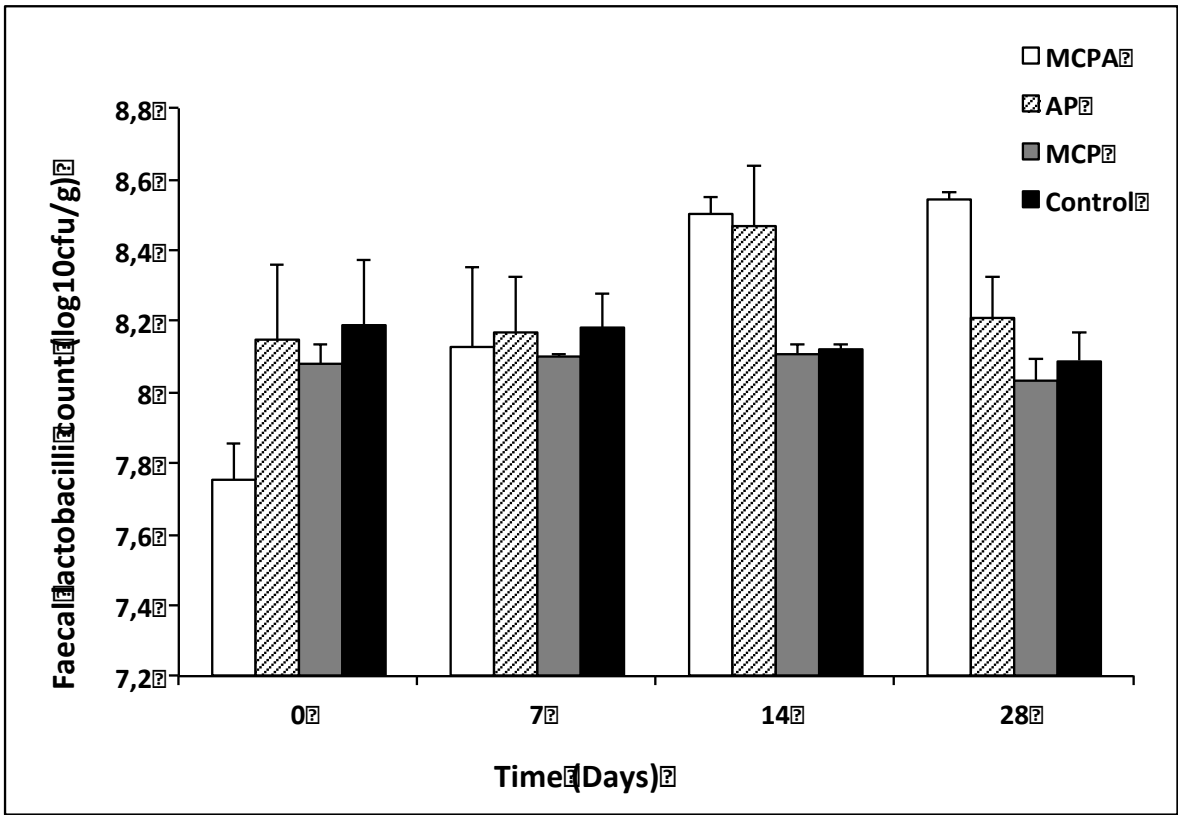

Figure 6: Average count of faecal lactobacilli ($\log_{10}$cfu/g of feaces) in colon tumour induced Balb/c mice treated with MCPA (modified citrus pectin alginate probiotic microbeads), AP (alginate calcium probiotic microbeads) and MCP (modified citrus pectin solution). Each column represents mean ± standard deviation (n=3).

Generic-Specific Identification of Faecal Bacteria by 16S rRNA Amplification

Henningsson et al. (2002) and Biagi et al. (2010) suggested pectin stimulates bacteria other than lactic acid bacteria. In our study, the DNA sample fragments encoding 16S region shows *Lactobacillus* spp., *Bacillus* sp. and *Enterococcus faecium* were present in faecal samples from the MCPA-, AP probiotic and control groups (Table 1). In the MCPA probiotic-treated mice, three of the DNA samples encoding 16S rRNA gene were closest to the genus *Lactobacillus* (*L. acidophilus*, *L. reuteri* and *L. johnsonii*). In the AP probiotic- and MCP treated groups; two and one *Lactobacillus* was found respectively. The rest belonged to other genera including *Bacillus and Enterococcus faecium* (both in MCPA, AP and MCP groups of mice) and one *Bifidobacterium* (MCP only).

The detection of significant alignment of DNA fragments encoding 16S region of the probiotic strain, *Lactobacillus acidophilus* ATCC 4356 was found only in both the MCPA and AP probiotic treated groups. In the MCP treated group, *Bacillus* sp. was not detected but the genus *Bifidobacterium* was found only in this group. This demonstrates that MRS agar is neither ideal nor reliable to quantify bifidobacteria in mice faeces. The blast hits showed high significant alignments (with Expected values 0.00) and regions of 16S rRNA gene homology of *Bacillus* sp. in both the MCPA and AP probiotic treated groups than in the control group, despite mismatched genes and gaps in some cases.

http://dx.doi.org/10.4314/ajtcam.v13i2.13

Table 1: Identification of faecal bacteria by genome sequence BLAST search

Treatment groups	Frequency of significant alignments	Genome sequence identified	Sequence identities (%)	Scores (Bits)	GenBank accession number
MCPA	2	*Lactobacillus acidophilus ATCC 4356*	100	2800	AB008203.1
	5	*Lactobacillus acidophilus La-14*	100	2784	CP005926.2
	5	*Lactobacillus acidophilus NCFM*	100	2784	CP000033.3
	6	*Lactobacillus reuteri TD1*	100	2766	CP006631.1
	6	*Lactobacillus reuteri I5007*	100	2766	CP006011.1
	6	*Lactobacillus reuteri DSM 20016*	99	2750	CP000705.1
	6	*Lactobacillus johnsonii NCC533*	100	2695	AE017198.1
	6	*Enterococcus faecium Aus0085*	100	2759	CP006620.1
	12	*Bacillus cereus*	100	1810	CP004870.1
AP	1	*Lactobacillus acidophilus ATCC 4356*	100	2800	AB008203.1
	5	*Lactobacillus acidophilus La-14*	100	2784	CP005926.2
	5	*Lactobacillus casei*	100	2800	JN560 892.1
	6	*Enterococcus faecium D.O*	100	2744	CP003583.1
	6	*Enterococcus faecium Aus0085*	100	2736	CP006620.1
	6	*Enterococcus faecium Aus0004*	100	2736	CP003351.1
	6	*Enterococcus faecium NRRL B-2354*	99	2736	CP004063.1
	12	*Bacillus cereus*	100	1703	CP008712.1
MCP	1	*Bifidobacterium sp*	99	2758	JF519689.1
	5	*Lactobacillus reuteri ATCC55730*	99	2758	EU394679.2
	6	*Enterococcus faecium D.O*	100	2744	CP003583.1
CONTROL	1	*Bacillus sp*	100	2760	KF870418.1
	4	*Lactobacillus reuteri*	99	2764	CP000705.1
	6	*Enterococcus faecium Aus0004*	100	2736	CP003351.1
	6	*Enterococcus faecium D.O*	100	2744	CP003583.1

MCPA - modified citrus pectin alginate probiotic microbeads, AP - alginate calcium probiotic microbeads and MCP – modified citrus pectin solution.

Conclusion

The increase in number of identified faecal bacteria and *Lactobacilli sp* in the MCPA probiotic treated mice shows that MCPA combined with resistant starch and *Lactobacillus acidophilus ATCC 4356* improves the stimulation and growth of colonic microflora. The results obtained in this study suggest that the novel MCPA microbeads are useful for effective oral delivery of bacterial cells and other bioactive compounds. MCPA can be used in probiotic therapy which may improve the prevention of colon cancer.

Acknowledgement

Financial support was provided by the Research department, Durban University of Technology, South Africa and Dr L. Reddy (personal contribution).

References

1. Anal, A. K. and Singh, H. (2007). Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci Tech. 18: 240-251.
2. Annan, N. T., Borza, A. D. and Hansen, L. T. (2008). Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. Food Res Int. 41: 184-193.
3. Biagi, G., Cipollini, I., Grandi, M. and Zaghini, G. (2010). Influence of some potential prebiotics and fibre-rich foodstuffs on composition and activity of canine intestinal microbiota. Animal Feed Sci Technol. 159: 50-58.
4. Bissahoyo, A., Pearsall, R. S., Hanlon, K., Amann, V., Hicks, D., Godfrey, V. L. and Threadgill, D. W. (2005). Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet. Toxicol Sci 88: 340-345.
5. Brinques, G. and Ayub, M. (2011). Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. J Food Eng, 103: 123-128.
6. Capurso, G., Marignani, M. and Fave, G. D. (2006). Probiotics and the incidence of colorectal cancer: when evidence is not evident. Dige Liver Disease 38, Supplement 2: S277-S282.
7. Chávarri, M., Marañón, I., Ares, R., Ibáñez, F. C., Marzo, F. and Villarán, M. D. C. (2010). Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. Int J Food Microbiol. 142: 185-189.

http://dx.doi.org/10.4314/ajtcam.v13i2.13

8. Chen, J. and Huang, X.-F. (2009). The signal pathways in azoxymethane-induced colon cancer and preventive implications. Cancer Biol Ther. 8: 1313-1317.
9. Chen, M.-J. and Chen, K.-N. (2007). Applications of Probiotic Encapsulation in Dairy Products. Encapsulation and Controlled Release Technologies in Food Systems. Blackwell Publishing.
10. Clark, P. and Martin, J. (1994). Selection of Bifidobacteria for use as dietary adjuncts in cultured dairy foods. III. Tolerance to simulated bile concentrations of human small intestines. Cult Dairy Prod J (USA).
11. DíAz-Rojas, E. I., Pacheco-Aguilar, R., Lizardi, J., Argüelles-Monal, W., Valdez, M. A., Rinaudo, M. and Goycoolea, F. M. (2004). Linseed pectin: gelling properties and performance as an encapsulation matrix for shark liver oil. Food Hydro. 18: 293-304.
12. Dinakar, P. and Mistry, V. (1994). Growth and viability of *Bifidobacterium bifidum* in cheddar cheese. J Dairy Sci. 77: 2854-2864.
13. FAO (2002). WHO working Group report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada 30.
14. Gbassi, G. K., Vandamme, T., Yolou, F. S. and Marchioni, E. (2011). *In vitro* effects of pH, bile salts and enzymes on the release and viability of encapsulated *Lactobacillus plantarum* strains in a gastrointestinal tract model. Int Dairy J. 21: 97-102.
15. Gebara, C., Chaves, K. S., Ribeiro, M. C. E., Souza, F. N., Grosso, C. R. F. and Gigante, M. L. (2013). Viability of Lactobacillus acidophilus La5 in pectin–whey protein microparticles during exposure to simulated gastrointestinal conditions. Food Res Int. 51: 872-878.
16. Gombotz, W. R. and Wee, S. F. (2012). Protein release from alginate matrices. Adv Drug Del Rev. 64, Supplement: 194-205.
17. Hansen, L. T., Allan-Wojtas, P. M., Jin, Y. L. and Paulson, A. T. (2002). Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. Food Microbiol. 19: 35-45.
18. Henningsson, Å. M., Björck, I. M. E. and Nyman, E. M. G. L. (2002). Combinations of indigestible carbohydrates affect short-chain fatty acid formation in the hindgut of rats. J Nutr. 132: 3098-3104.
19. Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S. and Razavi, S. H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chem. 111: 50-55.
20. Mandal, S., Puniya, A. K. and Singh, K. (2006). Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. Int Dairy J. 16: 1190-1195.
21. Maxwell, E. G., Belshaw, N. J., Waldron, K. W. and Morris, V. J. (2012). Pectin - an emerging bioactive food polysaccharide. Trends Food Sci Technol. 24: 64-73.
22. Mitropoulou, G., Nedovic, V., Goyal, A. and Kourkoutas, Y. (2013). Immobilization Technologies in Probiotic Food Production. J Nutr Metabol. 2013: 1 - 15.
23. Morris, V. J. (2009). Pectin galactans, galectins and health. Bioactive roles for pectin. AgroFOOD industry hi-tech, March/April 20: 37-40.
24. Mountzouris, K. C., Balaskas, C., Fava, F., Tuohy, K. M., Gibson, G. R. and Fegeros, K. (2006). Profiling of composition and metabolic activities of the colonic microflora of growing pigs fed diets supplemented with prebiotic oligosaccharides. Anaerobe 12: 178-185.
25. O'keefe, S. J. D., Chung, D., Mahmoud, N., Sepulveda, A. R., Manafe, M., Arch, J., Adada, H. and Van Der Merwe, T. (2007). Why Do African Americans Get More Colon Cancer than Native Africans? J Nutr. 137: 175S-182S.
26. Ooi, L.-G. and Liong, M.-T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. Int J Mol Sci. 11: 2499-2522.
27. Ortakci, F., Broadbent, J. R., Mcmanus, W. R. and Mcmahon, D. J. (2012). Survival of microencapsulated probiotic *Lactobacillus paracasei* LBC-1e during manufacture of Mozzarella cheese and simulated gastric digestion. J Dairy Sci. 95: 6274-6281.
28. Ortakci, F. and Sert, S. (2012). Stability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in yogurt and in an artificial human gastric digestion system. J Dairy Sci. 95: 6918-6925.
29. Pillay, V. and Fassihi, R. (1999). *In vitro* release modulation from crosslinked pellets for site-specific drug delivery to the gastrointestinal tract: II. Physicochemical characterization of calcium–alginate, calcium–pectinate and calcium–alginate–pectinate pellets. J Controlled Release 59: 243-256.
30. Sandoval-Castilla, O., Lobato-Calleros, C., García-Galindo, H. S., Alvarez-Ramírez, J. and Vernon-Carter, E. J. (2010). Textural properties of alginate–pectin beads and survivability of entrapped Lb. casei in simulated gastrointestinal conditions and in yoghurt. Food Res Int. 43: 111-117.
31. Sheu, T. Y. and Marshall, R. T. (1993). Microentrapment of Lactobacilli in calcium alginate gels. J Food Sci. 58: 557-561.
32. Shi, L.-E., Li, Z.-H., Li, D.-T., Xu, M., Chen, H.-Y., Zhang, Z.-L. and Tang, Z.-X. (2013). Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. J Food Eng. 117: 99-104.
33. Tanaka, T. (2009). Colorectal carcinogenesis: review of human and experimental animal studies. J carcinogenesis 8: 5.
34. Trabelsi, I., Bejar, W., Ayadi, D., Chouayekh, H., Kammoun, R., Bejar, S. and Ben Salah, R. (2013). Encapsulation in alginate and alginate coated-chitosan improved the survival of newly probiotic in oxgall and gastric juice. Int J Biol Macromol. 61: 36-42.
35. Trindade, C. F. and Grosso, C. (2000). The effect of the immobilisation of *Lactobacillus acidophilus* and *Bifidobacterium lactis* in alginate on their tolerance to gastrointestinal secretions. Milchwissenschaft 55: 496-499.