ICONIET 2018

Proceedings of the International Conference on Innovation, Entrepreneurship and Technology,
30-31 October 2018, BSD City, Indonesia,
ISSN: 2477-1538

Reformulation of Mice Fodder with Encapsulated *Lactobacillus fermentum* E5

Marcelia Sugata[a*], Andrew Jounathan[a] & Tjie Jan Tan[a]

[a]*Department of Biology, Universitas Pelita Harapan, Tangerang, Indonesia*

*marcelia.sugata@uph.edu

Abstract: Common in vivo probiotic delivery through oral gavage may result in esophageal injury as well as restraint-associated-distress particularly with repeated application. To overcome the issue, in this study, *Lactobacillus fermentum* E5 was embedded in sodium alginate-chitosan capsules and incorporated with commercial mice fodder. The survival of probiotic following various procedures and storage was evaluated. More than 90% of viable cells were successfully recovered from fresh microcapsules and 95% of the encapsulated probiotics survived freeze drying process. Furthermore, the encapsulated cells exhibited survivability of more than 85% after 28 days storage period at 4°C and room temperature. Reformulation of mice fodder was done by crushing the commercial pellet, adding encapsulated probiotic, mixing, re-pelleting using 3% (v/v) sodium alginate solution as binder. After storing at room temperature, almost 80% of encapsulated *L. fermentum* E5 in mice fodder survived.

Keywords: *Lactobacillus fermentum*, mice fodder, microencapsulation, reformulation

1. Introduction

Since the interest of consumers in food products containing probiotics has risen, the number of study related to the subject has been growing rapidly (Jankovic *et al*., 2010). Probiotic was defined as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). Lactic acid bacteria (LAB) is one of the most widely used probiotics in fermented foods, and many of them are considered as Generally Recognize as Safe (GRAS) (Alvarez-Sieiro *et al*., 2016). In human, LAB could be found in the oral cavity, gastrointestinal tract, and in the vagina (Duar *et al*., 2017).

Recent studies on LAB confirmed their health benefits in the prevention and treatment of inflammatory bowel disease and other related disease (Saez-Lara *et al*., 2015). Among the LAB, *Lactobacillus fermentum* has been reported for its ability to confer health benefit on host. Aoudia *et al.* (2015) found that feeding zebrafish embryos with *L. fermentum* significantly impaired the secretion of the pro-inflammatory cytokines IL-1β and TNF-α, and promoted the secretion of IL-10. Anderson *et al.* (2016) reported the ability of *L. fermentum* in increasing the number of macrophages and lymphocytes in germ-free rat colon. Rodriguez-Nogales *et al.* (2017) also revealed that *L. fermentum* could inhibit the increased expression of IL-2 and iNOS, which are considered as important inflammatory mediators of innate and/or adaptive immunity.

The immune-stimulating effect of LAB has been investigated using animal experimental model. Common in vivo probiotic delivery was done through oral gavage method (Anderson *et al*., 2016; Rodriguez-Nogales *et al*., 2017). However, the technique may result in esophageal injury as well as restraint-associated-distress particularly with repeated application (Brown *et al*., 2000). Regardless of the type of study being conducted, any source of external stress on mice could make data interpretation difficult and confound measured outcomes (de Meijer *et al*., 2010; Walker *et al*., 2012). Besides, mortality following repeated oral gavage is a widespread issue across all strains of mice, regardless of age or genetic background (Arantes-Rodrigues *et al*., 2012). Since the standard route of oral delivery is used in most preclinical study, these issues may become significant concern.

In this study, a formulation to incorporate encapsulated probiotic into a commercial mice fodder was developed, with expectation that tested mice would voluntarily consume the formulated

fodder when it is placed into their cage. Prior reformulation, the survival of probiotic during microencapsulation and freeze-drying procedure, as well as its shelf-life, was evaluated.

2. Materials and Methods

2.1. Culture preparation

L. fermentum E5 obtained from Universitas Pelita Harapan was used as the active material entrapped in microcapsules. The cells were inoculated in MRS broth (de Man, Rogosa, Sharpe) for 24 h under microaerophilic condition at 37°C, followed by harvesting by centrifugation at 8,000 rpm for 10 min at room temperature (RTP). The cultures, refer as free suspended cells, were then washed twice by sterile phosphate saline buffer and used in microencapsulation procedures (Mokarram *et al*., 2009).

2.2. Microencapsulation and coating procedure

Microcapsules were made according to a modified version of methods reported by Krasaekoopt *et al.* (2004). Sodium alginate (Merck) was used as encapsulation material. One ml of liquid culture was incorporated into 10 ml of 3% (w/v) sodium alginate solution. For coating agent, chitosan was dissolved in acidified distilled water to achieve final concentration of 0.75% (v/v). Chitosan aqueous solution was then mixed with 0.4 M calcium chloride solution (4% v/v).

To form microcapsules, probiotic-alginate solution was extruded drop-wise by using a syringe into stirred calcium chloride -chitosan solution. After 30 min at RTP, the completely solidify beads (fresh capsules) were sieved from calcium chloride -chitosan solution. One part of capsules was immediately used for enumeration, while the remaining part was freeze-dried.

2.3. Encapsulation yield

Entrapped bacteria in fresh capsules were released by homogenizing one gram of microcapsules in 9 ml of phosphate saline buffer for 1 h and stirred. The homogenized samples were diluted to appropriate concentrations and spread plated on MRS agar. The plates were incubated for 48 h at 37°C and viable cells were enumerated as CFU/ml (Chavarri *et al*., 2010).

Encapsulation yield (EY) which is measurement of the number of successfully entrapped bacteria, was calculated as Eq. (1).

$$EY\ (\%) = (N/N_0) \times 100 \quad (1)$$

where N is the number of viable entrapped cells released from the microcapsules, and N_0 is the number of free cells added to the alginate solution during microcapsules production.

2.4. Survival of L. fermentum E5 after freeze – drying

Fresh capsules were frozen at -30°C for 24 h and dried in freeze dryer Martin Christ Alpha 1-2 LD Plus (John Morris, Germany) for 48 h (0.63-0.47 mbar, 15-20°C) until freeze-dried capsules were obtained. To investigate the survival of bacteria after freeze-drying, freeze-dried capsules (0.1 g) were dissolved in 9 ml phosphate saline buffer and stirred. After 1 h at RTP, the released viable cells were enumerated by spread plating appropriate dilutions of dissolved capsules (Xu *et al*., 2016). Survivability of *L. fermentum* E5 after freeze-drying was calculated by applying Eq. (2).

$$Survivability\ (\%) = (N_1/N) \times 100 \quad (2)$$

where N_1 is the number of viable cell in freeze-dried capsules, and N is the number of viable cell in fresh capsules.

2.5. Reformulation of mice fodder and encapsulated L. fermentum E5

Mice fodders were obtained from PT. Charoen Popkhand. To do reformulation, twenty parts of fodders were crushed and mixed well with one part of encapsulated *L. fermentum* E5. Sodium alginate solution of 3% (v/v) was then added as a binder, followed by drying at 60°C for 30-180 min. Shelf-life of reformulated mice fodders was tested during 28 days of storage.

Proceedings of the International Conference on Innovation, Entrepreneurship and Technology,
30-31 October 2018, BSD City, Indonesia,
ISSN: 2477-1538

2.6. Storage of encapsulated L. fermentum E5

To investigate the shelf-life of encapsulated bacteria during storage period, the freeze-dried capsules were placed into test tubes and stored at RTP (±25°C) and in a fridge (4°C) for freeze-dried capsules. Meanwhile, reformulated mice fodders in closed petri dish was incubated at RTP. On the 14th and 28th of storage, the capsules (0.1 g for fresh capsules and 1 g for dried capsules) or reformulated mice fodders (1 g) were dissolved, diluted, and spread plated on MRS agar plate. Enumeration was done after 48 h of incubation at 37°C. The survival rate of bacteria during storage was calculated as Eq. (3).

$$\text{Survival rate} = N_2/N_1 \quad (3)$$

where N_2 is the number of viable cell in freeze-dried capsules, and N is the number of viable cell in freeze-dried capsules after storage period.

2.7. Statistical analysis

Data were presented as mean ± standard deviation ($n \geq 3$) and analyzed by one-way ANOVA. Statistical significance was set at 95% confidence interval ($p < 0.05$). All analyses were performed using ISI Applets for multiple means of quantitative response (Introduction to Statistical Investigations, Wiley, USA).

3. Results and Discussion

In this study, *L. fermentum* E5 cells were entrapped in alginate-chitosan capsules via the combination of gelation and extrusion technique. As the oldest and most common approach to making capsules with hydrocolloids, extrusion technique is easy, simple, and low cost. Besides, the technique provides gentle formulation conditions, ensuring high retention of cell viability (Krasaekoopt *et al*., 2003). Microcapsules of *L. fermentum* E5 before freeze drying was relatively sphere and uniform (Figure 1).

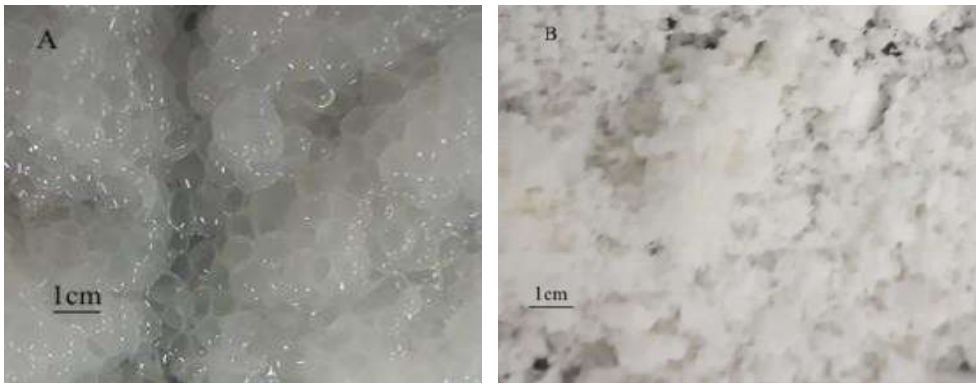

Figure 1. Microencapsulated cells before (A) and after (B) freeze-drying.

3.1. Encapsulation yield

Microencapsulation procedure caused no significant loss of viability for *L. fermentum* E5 and approximately 94% of cells were successfully entrapped (Table 1). In agreement with Krasaekoopt *et al.* (2003), the mean of encapsulation yield was 80-95% in extrusion technique. Furthermore, enumeration of viable cells showed that the number of free and entrapped cells remained significantly unchanged. According to Koo *et* al. (2001), a slight decrease in cell number after encapsulation process might occur due to very low pH caused by acetic acid glacial used to dissolve chitosan. The process of dissolving the microcapsules rather than the act of encapsulation itself might also reduce cell viability. To release entrapped cells from fresh capsules prior enumeration, microcapsules were broken down by vigorous stirring which most likely caused the physiological damage of the cells.

Table 1: Encapsulation yield and survival of *L. fermentum* E5 after freeze-drying

Free cells in suspension (log CFU/ml)	Viable cells in fresh capsules (log CFU/ml)	Viable cells in dried capsules (log CFU/ml)	Encapsulation yield (%)	Survival after freeze-drying (%)
11.49 ± 0.73^{a}	10.78 ± 0.24^{ab}	10.32 ± 0.40^{b}	93.99±4.09	95.70±3.01

Different letter between columns indicates significantly different values ($p<0.05$) according to one-way ANOVA. Data are presented as mean ± SD (n=4).

3.2. Survival of L. fermentum E5 after freeze - drying

Freeze-drying produced large and brittle granules with irregular structure (Figure 1). Referring to Cook *et al.* (2011), diffusion setting process during gelation may result in a higher polymer concentration on the surface of the capsules. As the capsule dries, the interior with a lower polymer concentration could shrink into a greater extent than the exterior regions, causing a crumpled structure with decreased size but relatively unreduced surface area. There was a 0.46 log decrease in viable cells associated with the drying process (Table 1), which could possibly be improved if cryoprotectants were used. Statistical analysis showed that double coating alginate-chitosan had a significant impact on the residual viability during freeze drying since the number of viable cells decreased insignificantly. As reported by Koo *et al.* (2001), alginate-chitosan could protect the cells from cold damage during freezing and freeze-drying process.

3.3. Shelf-life analysis of encapsulated L. fermentum E5

The stability of non-encapsulated and encapsulated cells during storage at 4°C and RTP is shown in Figure 2. The viable count of encapsulated cells remained stable through the storage period regardless temperature storage. In contrast, the number of non-encapsulated cells experienced a significant decrease at longer storage period. The high survivability of encapsulated cells was resulted from the protection provided by double coating alginate-chitosan. Correspondingly, previous study by Cook *et al.* (2011) found that encapsulating cells in alginate chitosan could promote greater cell survival.

After 28 days of storage at 4°C, there was a small loss of viability (~1-2 log) in both free and coated cells. Meanwhile, the number of cells placed at RTP decreased more than 6 log. The results suggested that the viability of encapsulated and non-encapsulated cells was higher while stored in low temperature. According to Holkem *et al*. (2016), storing microorganisms at a higher temperature, approaching its optimum growing temperature, would induce its metabolic activities which caused lower survivability. Besides, chemical reactions were reduced at low temperatures close to 0°C, thus the possibility of cell damage would be reduced (Xu *et al.*, 2016).

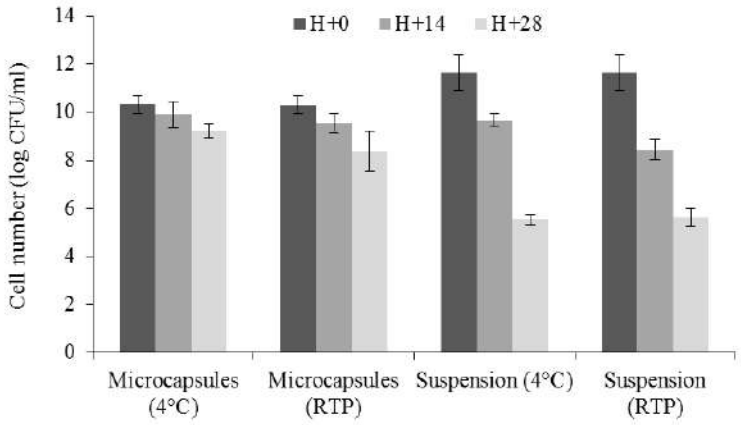

Figure 2. Survival of *L. fermentum* E5 at different storage temperature in forms of free suspended and encapsulated cells (n=4, error bars represent standard deviation between replications).

3.4. Reformulation of mice fodder

Reformulation was done by adding encapsulated probiotic to commercial mice fodder. After mixing the crushed fodder with microcapsules, it was re-pelleting using a self-made tool, as shown in Figure 3. Pressure was given manually, causing the shape of the reformulated pellets were not completely uniform (Figure 4). However, the diameter of the pellets is always similar. Alginate was used as a binder because it is cheap and does not give any additional nutrition value to the fodder (Rychen *et al*., 2017). For further research, improvements need to be done, especially in reformulation procedure and the usage of the tools.

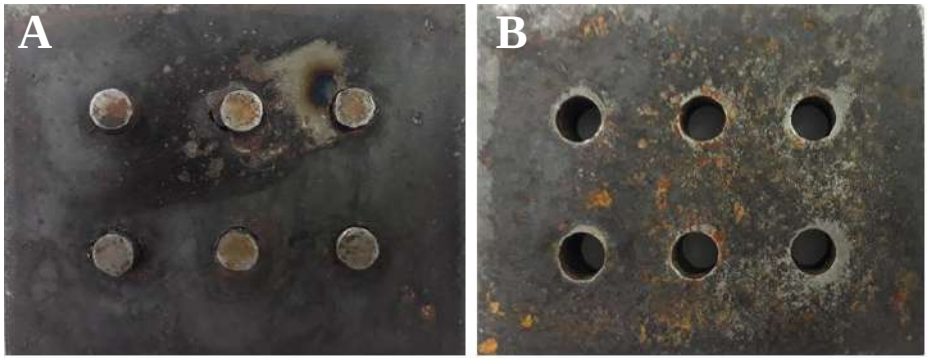

Figure 3. Re-pelleting tools (A) upper side and (B) bottom side.

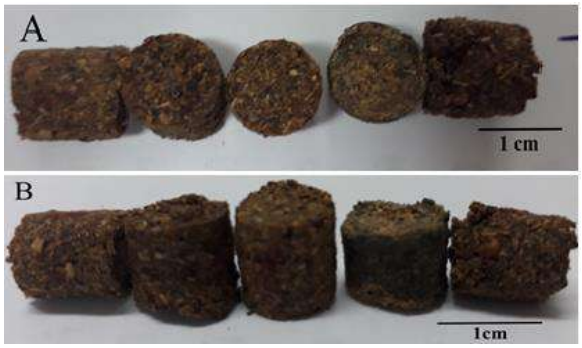

Figure 4. Reformulated mice fooder from (A) top view and (B) side view

3.5. Shelf analysis of encapsulated L. fermentum E5 *in reformulated mice fodder*

Since it is recommended that probiotic be present at a minimum level of 10^6 CFU/g of food product (Doleyres & Lacroix, 2005) or 10^7 CFU/g at point of delivery (Lee & Salminen, 1995) or be eaten in adequate amount to yield a daily intake of 10^8 CFU (Lopez-Rubio *et al*., 2006), a 28-day shelf-life analysis of encapsulated *L. fermentum* E5 incorporated in mice fodder was conducted (Figure 5). The viability of the encapsulated cells incorporated in mice fodder showed the same manner as in non-incorporated ones. The number of viable cells was further decreased with increasing storage period at RTP. In the first 14 days of storage, there was a slight loss of cell viability, initial numbers of 9.70 to 9.01 log CFU/g mice fodder. After 28 days of storage, cell survival decreased significantly to 8.30 log CFU/g, as compared to the initial number. These results suggested that alginate-chitosan could provide cell stability in mice fodder during storage at RTP.

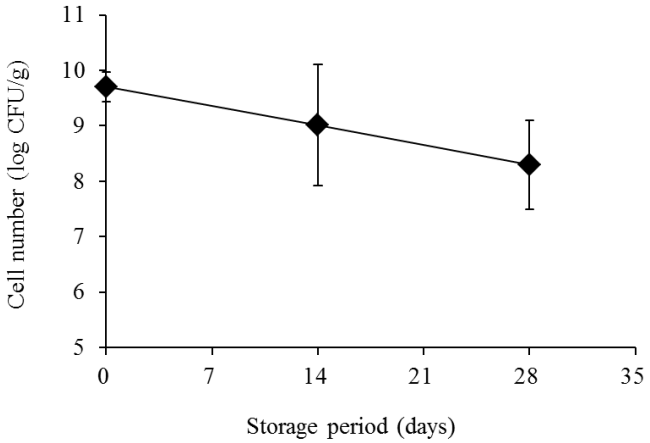

Figure 5. Survival of encapsulated *L. fermentum* E5 incorporated in mice fodder stored at room temperature (n=4, error bars represent standard deviation between replications).

4. Conclusion

After microencapsulation procedure, 94% of free suspended *L. fermentum* E5 were successfully entrapped and 95% of the encapsulated cells recovered from freeze drying process. Shelf-life analysis showed that encapsulated cells had higher survival than free cells regardless temperature storage. It is concluded that alginate chitosan was no harm for encapsulation procedure and effectively protect the cells during freeze-drying process and storage period. Reformulation was done accordingly, but re-pelleting process needs to be improved for further study. After storing encapsulated *L. fermentum* E5 incorporated in mice fodder at room temperature for 28 days, the cells of more than 10^8 CFU survived. Further study is needed to test the hypothesis that the reformulated mice fodder could be used as less stressful oral delivery method as compared to oral gavage.

Acknowledgements

This study was conducted in Fundamental Biology Laboratorium (202), Advanced Biology Laboratorium (407) and Food Research and Development Laboratorium (211) at Universitas Pelita Harapan. The authors would like to thank Universitas Pelita Harapan (UPH), Faculty of Science and Technology (FaST), Biology Department, and Food Technology Department for their support. Special appreciation to Head of Biology Department, Head of Advanced Biology Laboratory, Head of Fundamental Biology Laboratory, and Center for Research and Community Development (LPPM) UPH.

References

Alvarez-Sieiro, P., Montalban-Lopez, M., Mu, D., and Kuipers, O.P. (2016) Bacteriocins of lactic acid bacteria: extending the family. Applied Microbiology and Biotechnology, 100, 2939-2951.

Anderson, R.C., Ulluwishewa, D., Young, W., Ryan, L.J., Henderson, G., Meijerink, M., Maier, E., Well, J.M., and Roy, N.C. (2016) Human oral isolate Lactobacillus fermentum AGR1487 induces a pro-inflammatory response in germ-free rat colons. Scientific Reports, 6, 203-218.

Aoudia, N., Rieu, A., Briandet, R., Deschamps, J., Chluba, J., Jego, G., Garrido, C., and Guzzo, J. (2015) Biofilms of Lactobacillus plantarum and Lactobacillus fermentum: effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. Food Microbiologu, 53, 51-59.

Arantes-Rodrigues, R.R., Henriques, A.A., Pinto-Leite, R.R., Faustino-Rocha, A.A., Pinho-Oliviera, J.J., Teixeira-Guedes, C., Seixas, F., Gama, A., Colaco, B., Colaco, A., and Oliveira, P. A. (2012) The effects of repeated oral gavage on the health of male CD-1 mice. Lab Animal (NY), 41(5),129–134.

Brown, A.P., Dinger, N., and Levine, B.S. (2000) Stress produced by gavage administration in the rat. Contemporary Topics in Laboratory Animal Science, 39, 17–21.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., and Khutoryanskiy, V. (2011) Production and Evaluation of Dry Alginate-Chitosan Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria. Biomacromolecules, 12, 2834-2840.

De Meijer, V. E., Le, H. D., Meisel, J. A., and Puder, M. (2010) Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. Physiology & Behavior,100:387–393.

Doleyres, Y., and Lacroix, C. (2005) Technologies with free and immobilised cells for probiotic bifidobacteria production and protection. International Dairy Journal, 15, 973–988.

Duar, R. M., Lin, X. B., Zheng, J., Martino, M. E., Grenier, T., Perez-Munoz, M. E., Leulier, F., Ganzle, M.,and Walter, J. (2017) Lifestyles in transition: evolution and natural history of the genus Lactobacillus. FEMS Microbiology Reviews, 41, S27-S48.

Fuller, R. (1989) A review: Probiotics in man and animals. Journal of Applied Bacteriology, 66, 365-378.

Holkem, A. T., Raddatz, G. C., Nunes, G. L., Chichoski, A. J., Lopes, E. J., Grosso, C. R. F., and Menezes, C. R. (2016) Development and characterization of alginate microcapsules containing Bifidobacterium BB-12 produced by emulsification/internal gelation followed by freeze drying. Food Science and Technology, 71, 302-308.

Jankovic, I., Sybesma, W., Phothirath, P., Ananta, E., and Mercenier, A. (2010) Application of probiotics in food products—challenges and new approaches. Current Opinion in Biotechnology, 21 (2), 175–181.

Koo, S.M., Cho, Y.H., Huh, C.S., Baek, Y.J., and Park, J., (2001) Improvement of the Stability of Lactobacillus casei YIT 9018 by Microencapsulation Using Alginate and Chitosan. Journal of Microbiology and Biotechnology, 11(3), 376-383.

Krasaekoopt, W., Bhandari, B., and Deeth, H. (2003) Evaluation of encapsulation techniques of probiotics for yogurt. International Dairy Journal, 13, 3-13.

Krasaekoopt W., Bhandari B., and Deeth H. (2004) The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. International Dairy Journal, 14, 737–743.

Lee, J., Yun, H. S., Cho, K. W., Oh, S., Kim, S. H., Chun, T., Kim, B., and Whang, K. Y. (2011) Evaluation of

Lee, Y.K., and Salminen, S. (1995) The coming to age of probiotics. Trends in Food Science & Technology, 6, 241–245.

Lopez-Rubio, A., Gavara, R., and Lagaron, J.M. (2006) Bioactive packaging: turning foods into healthier foods through biomaterials. Trends in Food Science & Technology, 17, 567–575.

Mokarram R. R., Mortazavi, S. A., Najafi, M. B. H., and Shahidi, F. (2009) The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. Food Reserarch International, 42, 1040-1045.

Rodriguez-Nogales, A., Algieri, F., Garrido-Mesa, J., Vezza, T., Utrilla, M. P., Chueca, N., Garcia, F., Olivares, M., Rodriguez-Cabezas,E., and Galvez, J. (2017) Differential intestinal anti-inflammatory effects of Lactobacillus fermentum and Lactobacillus salivarius in DSS mouse colitis: impact on microRNAs expression and microbiota composition. Molecular Nutrition & Food Research, 61, 1700144-1700183.

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. D., Bories, G., and Gropp, J. (2017) Safety and efficacy of sodium and potassium alginate for pets, other non food-producing animals and fish. EFSA Journal, 15, 49-45.

Saez-Lara, M. J., Gomez-Llorente, C., Plaza-Diaz, J., and Gil, A. (2015) The Role of Probiotic Lactic Acid Bacteria and Bifidobacteria in the Prevention and Treatment of Inflammatory Bowel Disease and Other Related Diseases: A Systematic Review of Randomized Human Clinical Trials. BioMed Research International, 2015, 1-15.

Walker, M. K., Boberg, J. R., Walsh, M. T., Wolf, V., Trujillo, A., Duke, M. S., Palme, R., and Felton, L. A. (2012) A less stressful alternative to oral gavage for pharmacological and toxicological studies in mice. Toxicology and Applied Pharmacology, 260, 65-69.

Xu, M., Borque, F. G., Dumont, M. J., and Jabaji, S. (2016) Encapsulation of Lactobacillus casei ATCC 393 cells and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions. Journal of Food Engineering, 168, 52-59.