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TITLE: Production of Pelleted Feed Containing *Lactobacillus reuteri* KUB-AC5 by Cold Pelleting Process

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THESIS

PRODUCTION OF PELLETED FEED CONTAINING *LACTOBACILLUS REUTERI* KUB-AC5 BY COLD PELLETING PROCESS

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A Thesis Submitted in Partial Fulfillment of
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Lactobacillus reuteri KUB-AC5 isolated from chicken intestines is a potential probiotic supplement in chicken feed. *L. reuteri* KUB-AC5 incorporated into feed ingredients with various contents of modified starch (1%, 2% and 3% w/w) and fresh liquid culture (20%, 25% and 30% v/w) or spray dried *L. reuteri* KUB-AC5 (1%w/w) were mixed prior to the pelleting process at ambient temperature. The wet pellets were then hot air-dried at 40°C and 50°C. To obtain the pellet moisture of ca. 8%, the optimum drying times were 8 h and 4 h at 40°C and 50°C, respectively. The physical quality of the pellet was evaluated in term of pellet hardness. It was ranking of 55 to 107 N, depending on modified starch content. Pellet hardness was increased with increasing the modified starch content. Moreover, the addition of protective agents such as CMC and sodium alginate were found to increase pellet hardness (ca. 107 N for CMC and 95 N for sodium alginate). After drying at 40°C and 50°C, the viability of *L. reuteri* KUB-AC5 was decreased approximately 1-2 log cycles. The results also showed that pellet with 1% and 2% modified starch demonstrated the highest viable cell after drying at 40°C and 50°C, respectively. Furthermore, to enhance viability of the strain during drying, protective agents were added into the cell suspension. From results, pelleted feed with 20% milk powder showed the highest survival of 30.85% and 20.73% after drying at 40°C and 50°C, respectively. The viability of *L. reuteri* KUB-AC5 was also evaluated during storage in aluminum seal bag at 4°C and 30°C. During storage at 4°C for 150 days, the viability of all samples was quite stable with viable cell of ca. 10^7-10^8cfu/g depending on type of protective agent. While, the viability of the strain with and without protective agent during storage at 30°C was absolutely lost within 60 and 30 days, respectively. Similar results were observed when spray-dried *L. reuteri* KUB-AC5 was used. The viability of spray-dried *L. reuteri* KUB-AC5 was also quite stable during storage at 4°C and the 2% modified starch showed the highest survival (51.28% for 40°C and 27.58% for 50°C). During 30°C storage, the survival of the strain was also completely lost within 60 days.

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INTRODUCTION

For many decades, antibiotics/growth promoters have been used as feed additives in poultry farm to reduce the infection of pathogenic bacteria such as *Salmonella* sp. and *Escherichia coli*. Furthermore, most cases performance parameters such as body weight gain or feed conversion ratio improves (Simon, 2005). However, it was found that antibiotics linked to the emergence of multiple drug resistant bacteria. The presence of undesired antibiotic residues in meat has largely added to the public concerns regarding the use of antibiotics in the feed (Van Immerseel *et al*., 2002). From 1st January 2006 onwards, the European Union (EU) has decided to ban antibiotics as feed additives (Simon, 2005). Hence, probiotics have been considered for alternative to antibiotic. The use of probiotics as animal feed supplements dates back to the 1970s. They increase growth of animal and improve its health by increasing its resistance to disease and stimulating the immune system (Fuller, 1992). However, the health benefits will occur when probiotic strain reach the intestine in viable form and in sufficient numbers. Therefore, the survival of the probiotic is required during feed processing and storage.

Currently, the pelleted feed has been used in poultry farm in order to improve feed efficiency, reduce selective feeding and decrease feed wastage. However, the most commonly used probiotic contains strains of lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, which rarely produce optimum results in pelleted feed. It is most likely because the lactic acid bacteria are destroyed partly and totally by the current pelleting process. Normally, lactic acid bacteria have the optimum viability temperature in ranging of 30-37°C (Harun-ur-Rashid *et al*., 2007), while pelleting process may raise the temperature of finished feed up to 65-85°C (Petis *et al*., 2005). It is a great challenge to find a suitable method to produce feed containing probiotics without losses of viability. Hence, this thesis research will

develop the manufacturing process of pelleted feed containing probiotic to maintain viability of cell.

The objectives of this study were:

1. To investigate the optimum conditions to produce pelleted feed containing *Lactobacillus reuteri* KUB-AC5.
2. To evaluate the survival of *L. reuteri* KUB-AC5 in pelleted feed during drying and storage at various temperature.
3. To determine the effect of protective agents on cell viability.

LITERATURE REVIEW

1. Probiotics and their application in poultry

Probiotics have been suggested as alternative to the use of antibiotics in animal feed. According to Fuller (1989), probiotics are characterized as live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. The probiotic bacteria must be general regards as safe (GRAS), able to colonize the gastrointestinal tract, survive the low pH of the stomach and bile acids in the intestines, and complete against other microorganisms in the gastrointestinal tract (Ahmad, 2004; Kosin and Rakshit., 2006; Patterson and Burkholder, 2004). The microorganisms used in animal feed are mainly bacterial strains of gram positive bacterial belonging to the types *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Bacillus*. Some other probiotics are microscopic fungi such as strains of yeast belonging to the *Saccharomyces cerevisiae* species (Table 1). The most commonly used probiotics contains strains of lactic acid bacteria (*Lactobacillus*, *Bifidobacterium* and *Streptococcus*).

Table 1 Microorganisms used in probiotics preparation for farm animals

Lactobacillus	*Bacillus*	*Bifidobacterium*	Other species
L. acidophilus	*B. coagulans*	*B. animalis* subsp.	*Aspergillus oryzae*
L. brevis	*B. lentus*	*lactis*	*Enterococcus*
L. casei	*B. licheniformis*	*B. longum*	*aecium*
L. fermentum	*B. pumilus*		*Saccharomyces*
L. plantarum	*B. subtilis*		*cerevisiae*
L. reuteri			*Streptococcus*
L. rhamnosus			*faecium*
L. lactis			*S. thermophilus*

Source: Modified from European commission (2008)

Proposed mechanisms of pathogen inhibition by the probiotic microorganisms include competition for nutrients, production of antimicrobial conditions and compounds (volatile fatty acid, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Gibson and Fuller, 2000). Lactic acid bacteria have been demonstrated to inhibit the growth of many enteric bacteria (*Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Clostridium perfringens, and Closrtidium difficile*) and have been used in animals to treat a broad range of gastrointestinal disorders. Lactic acid bacteria against different pathogenic microorganisms related to the production of bacterial substances as bacteriocins, organic acids, and hydrogen peroxides (Graban, 2002). Bacteriocin is a group of potent antimicrobial peptides or proteins primarily active against the same or closely related species organisms by adsorption to receptors on the target cells, insertion and pore formation (Ouwehand and Vesterlund, 2004). Nisin is a typical bacteriocin produced by *Lactococcus lactis*. Nisin is active against gram positive bacteria such as, *Clostridium*, *Bacillus, Listeria* and *Staphylococcus* sp. (Jack *et al*, 1995). *Lactobacillus*, isolated from chicken intestine, exhibited the inhibition of *E. coli* and *Salmonella* sp. by production of organic acid (Jin *et al*., 1996) which creates an unsuitable environment for pathogens to grow (El-Naggar, 2004). Organic acids can diffuse the cell membrane, because of their fat solubility and dissociate inside the cell. Therefore, bacterial cell is reduced the intracellular pH and metabolic activities (Bearson *et al*., 1997). In addition, several lactobacillus strains have been found to produce various types of antibiotics such as *L. acidophilus* produces lactacin, while *L. plantarum* produces plantaricin (El-Naggar, 2004).

Probiotic as farm animal feed supplements have been used since the 1970s. Probiotics were originally incorporated into feed to increase the animal growth and improve its health by increasing the resistance to disease. Tortuero (1973) used poultry preparations containing living bacteria and observed that using of lactobacilli increased in growth rate in chicken and their performances were similar to those obtained when using antibiotics. A positive effect of the applied *Lactobacillus* on egg production was described by Nahashon *et al*. (1996). Pedroso *et al*. (1999) have reported that the use of probiotics (*B. subtilis*) improved feed conversion and eggshell

thickness. Improvement of these two factors will result in significantly improved profit margins for the egg producer.

The use of probiotics to improve productivity in poultry is currently generating a great deal of interest. They have been reported to have many beneficial effects when used in poultry feeds as a mean of controlling pathogen carriage, which include competitive exclusion of pathogen, improved digestion and absorption of nutrients. These have a positive effect on growth rate and feed conversion. According to Jin *et al.* (1998), the addition of either a single *L. acidophilus* I26 strain or a mixture of *Lactobacillus* cultures to the basal diet increased feed conversion ratio and body weight of broiler and decreased its morality. Similarly, broiler fed diets supplemented *L. acidophilus*, *L. casei* and *Scytalidium acidophilum* had body weight improved (Hueng *et al*., 2004). Moreover, the addition of probiotics in drinking water increased total final body weight and reduced the mortality of broilers (Timmerman *et.al*, 2006). This result was supported by Safalaoh (2006). Liquid of effective micro-organisms (EM; mixture of *L. plantarum, L. casei*, *Streptococcus. lactis*, *Rhodopseudomonas palustris, Rhodobacter sphaeroides, Saccharomyces cerevisiae, Candida utilis, Streptomyces albus, Streptomyces griseus, Aspergillus oryzae* and *Mucor hiemalis*) were added in drinking water of broiler. The result demonstrated that chickens received EM had higher body weight gains. In addition, the dressing percentage (carcass weight as a proportion of body weight) of EM supplemented chickens was higher than the control. Murry *et al*. (2006) found that the diets containing *Lactobacillus* supported the growth of broilers. It was not significantly difference with the basal diet supplemented antibiotic and coccidiostat. Recently, Mountzouris *et al.* (2007) investigated the efficacy of a new multibacterial species probiotic (2 *Lactobacillus* strains, 1 *Bifidobacterium* strain, 1 *Enterococcus* strain, and 1 *Pediococcus* strain) in broiler nutrition. They revealed that treatment of probiotic in feed and water (PFW) displayed a growth promoting effect that did not differ from antibiotic in feed (AB). Similarly, feed conversion ratio in treatment of AB and PFW were significantly better than the control treatment.

Furthermore, probiotic supplementation of the intestinal microflora in poultry, especially with *Lactobacillus* species, exhibited beneficial effects on resistance pathogenic and non-pathogenic. According to Jin *et al*. (1998), the coliform counts obtained from ileum and cecum of 10 days broiler which fed diets with *Lactobacillus* cultures had slightly lower. After 30 days feeding the lactobacilli population in cecum of broiler fed diets with either *L. acidophilus* or a mixture of Lactobacillus cultures were higher than broilers fed diets without Lactobacillus. Kralik *et al*. (2004) added *Enterococcus faecium* M-74 in drinking water of broiler and investigated the influence of the strain on the intestinal microflora composition**.** The result showed that the strain reduced bacteria from family Enterobacteriaceae*, E. coli, S. aureus* and *Enterococcus faecalis*. Similarly, Kaĉåniová *et al*. (2004) recorded that decreasing counts of *E. coli* were found in chickens sprayed with *Enterococcus faecium*. As well as counts of *E. coli* in turkeys decreased when they received the strain via drinking water once a week. Murry *et al*. (2006) reported that the diets supplemented with the botanical probiotic containing *Lactobacillus* reduced *C. perfringens* and *C. jejuni* in clocca and carcass of broilers. Gunal *et al*. (2006) found that the probiotic alone or a combination of probiotic with organic acid mixture added in diets decreased on ileal and caecal negative bacteria counts.

Commercial poultry production must receive some benefits in order to change prophylactic/growth promoting antibiotic use to the application of probiotic bacteria in its management programs. As mentioned earlier, probiotics can reduce the number of potentially pathogenic bacteria in the intestinal tract of production broilers, layers and turkeys. In healthy chicks little benefit can be seen in the use of probiotics, but in the real world of the broiler house and in the cage layer house, there are numerous potential challenges from bacteria and even protozoan parasites that will compromise the performance of poultry. Thus, use of probiotics has the potential for reduction of risk of infection from pathogens and also totally eliminates the possibility for induction of antibiotic resistance among pathogenic organisms. Furthermore, the potential for carcass contamination from gut-associated pathogens appears to be reduced and therefore public health concerns are decreased. If these were the only benefits that could be derived from the use of probiotics, it would be worth the cost of

change from prophylactic use of antibiotics. However, there are other benefits derived from the use of probiotics that affect the chicken. Specifically, immuno-stimulation as indicated by improved production of immunoglobulins IgA, IgM, and IgG, improved phagocytosis, improved cytokine production, promotion of natural killer T-cells, $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, and ultimately faster shed of intestinal pathogens. Dunham *et al*. (1993) reported that chickens treated with *L. reuteri* exhibited longer ileal villi and deeper crypts due to response associated with enhanced T cell function and increased production of anti-Salmonella IgM antibodies. *Lactobacillus* supplementation of layers diets increased cellularity of Peyer's patches in the ileum indicating a stimulation of the mucosal immune system that responded to antigenic stimuli by secreting immunoglobulin (IgA) (Nahanshon *et al*., 1996). Additionally, there is evidence in the literature to demonstrate improved growth and feed efficiency in chickens and in turkeys. The improved performance of chickens and turkeys fed probiotics can be correlated with microstructures in the intestine where villus height increased, goblet cell numbers increase, and crypt depth is decreased. Santin *et al*. (2001) showed that broiler fed with *Saccharomyces cerevisi*ae had villus height higher than control group. The broiler fed diets with Protexin (*L. acidophilus, L. plantarum, L. rhamnosus, L. bulgaricus, Streptococcus thermophilus, Aspergillus oryzae, B. bifidum, E. faecium, Candida pintolepesii*) had the increasing in jejunum and ileum villus height (Gunal *et.al*., 2006). In addition, the better performance, carcass yield and meat quality of broiler were improved when chickens fed diet containing probiotics and prebiotics (Takahashi *et al*., 2005). Thus, probiotics improve the morphology of the intestinal tract leading to improve absorption of nutrients. The cost of probiotics is competitive with the use of antibiotic growth promoters making them just as attractive as the growth promoters.

Use of probiotics as a routine practice in meat and egg producing poultry species has had a slow start, but it appears that increasing pressures from consumers will force the industry to adapt or fail. Adaptation simply means that antibiotics are no longer acceptable by the consumer of poultry products. If there is a small increase in the cost of production due to some alternative to antibiotic growth promoters, the consumer appears to be prepared to pay the additional cost. Market driven decisions

by corporate consumers of poultry products will also drive the poultry industry to adapt to a new standard of probiotics. Therefore, the benefit that the commercial poultry industry will derive from the change from antibiotic growth promoters to probiotics will be survival. Survival is then measured in terms of maintenance of market shares and continued domestic and international sales. However, the industry will also derive the benefit of improved welfare status of their chick. It is not uncommon to find that improved welfare also is associated with improved performance and improved profit margins. In time, it appears that antibiotic resistance factors might be lost from potentially zoonotic bacteria, and this would result in less difficulty in maintenance of flock health and public health of the consumers of poultry products. Therefore, the commercial poultry industry has numerous benefits to be gained from the use of probiotics and much to lose if it does not adapt to the new era where consumers tell producers how to produce the product that will be purchased.

2. Production of probiotic as feed additive

Probiotic preprations for feed applications are frequently supplied in frozen or dried form. Most liquid/frozen probiotic cultures require refrigeration for storage and distribution, thereby adding expense and inconvenience to their widespread use (Carvalho *et al*., 2004, Meng *et al*., 2008). Therefore, the dried form has been introduced because it would be easier handling, lower costs in storage and transportation (Santivarangkna *et al*., 2008). In particular, most dried starter cultures have been prepared by desiccation technology including freeze drying and spray drying.

2.1 Freeze drying

Freeze drying has been used to manufacture probiotic powders for decades and has been considered as a suitable dehydration process for bacteria, with the ultimate goal of achieving a solid and stable final formulation (Carvalho *et al*., 2004). The freeze drying is based upon sublimation, occurring in three phases; freezing, primary drying, and secondary drying. There are two ways to freeze microorganism

sample before drying, either within the freeze dryer chamber which is cooled by mechanical refrigeration dry ice and methanol, or liquid nitrogen (Carvalho *et al*., 2004) or the product can be frozen prior to loading onto the freeze dryer (Meng *et al*., 2008). During freezing, extracellular ice crystals usually form first and the solutes will be concentrated in a smaller volume of unfrozen water. The lower the temperature falls, the higher the solute concentration is. This higher extracellular osmotic pressure leads to the migration of cellular water outwards (Santivarangkna *et al*., 2008). The cell begins to dehydrate. Many articles reported that cellular inactivation occurs mostly at the freezing step. To and Etzel (1997) demonstrated that the survival of cell after freezing was 60 to 70%. The injury of cell during freezing step may relate to the thawing of cells. Cells must transverse a lethal intermediate zone of temperature (-15°C to -60°C) which is difficulty in viability determination in the frozen state (Santivarangkna *et al*., 2008). Furthermore, the increasing of electrolyte concentration and mechanical of ice formation may damage the cell membrane and intracellular components. The inactivation of cells in the freezing step depends on a cooling velocity. The slow freezing offers the survived cell lower than fast freezing because it may create the larger ice crystal formation which can cause damage to cell membranes (Morgan *et al*., 2006). Moreover, Fowler and Toner (2005) found that the fast freezing can avoid solute effects and excessive cellular shrinkage. However, if the freeze rate is too high, intracellular ice formation (IIF) will occur. A result of damages or defects is developing in cell membranes. Thus, an optimal freezing rate should be low enough to avoid IIF but high enough to minimize the solution effects (Santivarangkna *et al*., 2008). In addition, the injury of cell membrane by this mechanism cannot repair after desiccation and thus reducing cell viability (Morgan *et al*., 2006). The surface area of cell also had affected on the cell injury. It was found that the damage of membrane increased with increasing surface area of cell. Fonseca *et al.* (2000) demonstrated that streptococci as small spherical cells generally survived better than larger rod shaped of lactobacilli.

Follow by freeze drying process, there are comprised of two main parts primary and secondary drying phases. During the primary drying phase, the pressure is lowered and enough heat is supplied for elevating the temperature. The 95% water

in frozen is then removed by sublimation. It is important that the temperature in this phase should not be higher than collapse temperature which is the maximum temperature for preventing the structure of the dried product from macroscopic collapse (Morgan *et al*., 2006; Santivarangkna *et al*., 2008). Unfrozen water (4–5% g H_2O/g dry weight in fully hydrated cells) as bound water is subsequently removed in the secondary drying phase by desorption. The product temperature eventually is raised to an ambient temperature (Santivarangkna *et al*., 2008). Bound water is trapped within the solid matrix which is removed difficultly. Thus, the removal time will take extended periods (Mujumdar, 1995) and the temperature of this stage is raised higher than in the primary drying phase in order to break any physicochemical interactions formed between the water molecules and the frozen material (Morgan *et al*., 2006). Removal of bound water from bacterial cells during drying leads to damage of surface proteins, cell wall and the cell membrane because it has important role in stabilizing structural and functional integrity of biological macromolecules. Consequently, water removal during drying affects on destabilization of the structural integrity of cellular components which result in loss or impairment of function (Meng *et al*., 2008). Furthermore, van de Guchte *et al*. (2002) found that the efficacy of DNA replication, transcription and translation was reduced and the secondary structures of RNA and DNA destabilize during drying. For achieve grate results during the desiccation of probiotics, attention must be strongly focused on approaches to minimize harm of cellular components.

As mentioned above, freeze drying is the most widespread technique for dehydration of probiotic but this technique brings about undesirable side effects, such as damaged cell membrane, cell wall and other cellular components which are believed to be cause of cell inactivation. For successful delivery in feeds, probiotics must survive during production and maintain their biological function within the host. It is recommended that probiotic bacteria must be viable and available at a high concentration, typically 10^6–10^7 cfu/g of product (Kosin and Rakshit, 2006). Consequently, the choice of addition of cryoprotectants/protective agents is selected to help overcome cell inactivation during drying (Carvalho *et al*., 2004; Meng *et al*., 2008; Morgan *et al*., 2006; Ross *et al*., 2005). Protectants can be added either during

growth of the microorganism or prior to freezing or drying. Compatible cryoprotectants may be added to media prior to fermentation to assist in the adaptation of probiotics to the environment (Capela *et al*., 2006). As compatible cryoprotectants accumulate within the cells, the osmotic difference between the internal and external environments is reduced (Kets *et al*., 1996). The numerous cryoprotectants used in the preparation of microorganisms consists of a variety of simple and more complex chemical compounds as shown in Table 2. Nevertheless, only a few of them appear to work well for instant dimethylsulfoxide (Me_2SO), glycerol, blood serum or serum albumin, skimmed milk, peptone, yeast extract, saccharose, glucose, methanol, polyvinylpyrrolidone (PVP), sorbitol, and malt extract. The effective of protectant depends on species or strain of microorganism, cell size and form, growth phase, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell water content, lipid content and composition of the cells, storage temperature and duration of storage (Hubálek, 2003).

Skim milk powder is the most selected as protective agent for lactic acid bacteria (Carvalho *et al.*, 2004) because it is able to prevent cellular injury by stabilizing the cell membrane constituents, to create a porous structure in the freeze-dried product that makes rehydration easier and to contain proteins that provide a protective coating for the cells. Skim milk has often been used at a concentration of 1–10%, but even more frequently in the freeze drying, it will be combination with other protectances (Hubálek, 2003). Otero *et al.* (2007) found that the use of skim milk or skim milk-lactose or sucrose in *L. delbrueckii* suspension increased resistance to the freeze-drying process. Similarly, skim milk also enhanced survival of exponential-phase cells of *L. reuteri* during freeze-drying (Schwab *et al*., 2007). This result was supported by Huang *et al.* (2006). Skim milk was the best effective freeze-drying protective agent for *L. bulgaricus* LB14 and the optimal concentration was 130 g/l. Cell viability of the strain was up to 86.53% in this medium. Moreover, Berner and Viernstein (2006) found that maximum viability of *Lactococcus lactis* Sr. 3.54 (78%) was obtained after freeze-thawing with sucrose and skim milk mixtures as protective agents.

Table 2 Cryoprotectants used in microbiology

Chemical structure	Compounds
Sulphoxides	Dimethylsulfoxide
Monohydric alcohols and derivatives	Methanol, Ethanol, Polyvinyl alcohol
Diols and derivatives	Ethylene glycol, Propylene glycol, Trimethylene glycol, Diethylene glycol, Polyethylene glycol Polypropylene glycol, Polyethylene oxide
Triols	Glycerol
Polyalcohols	Mannitol, sorbitol, dulcitol
Monosaccharides	Glucose, Xylose
Disaccharides	Sucrose, Lactose, Maltose, Trehalose
Trisaccharides	Raffinose
Polysaccharides	Dextran, Mannan, Dextrin, Hydroxyethyl starch, Ficoll, Gum arabic (acacia)
Amides, N-alkylamides, imides	Acetamide, Methylacetamide, Dimethylformamide, Dimethylacetamide, Succinimide
Heterocyclic compounds	Methylpyrrolidone, Polyvinylpyrrolidone
Amino acids and carbonic acids	Proline, Glycine, Glutamic acid, monobutyric acid, Ammonium acetate, EDTA
Proteins, peptides, polypeptides, and glycoproteins	Blood serum, albumins, Gelatin, peptones, Shell extract, Glycoproteins, mucin,
Complex substrates	Yeast extract, Malt extract, Skimmed milk
Nonionic surfactants	Tween 80, Triton, macrocyclon

Source: modified from Hubálek (2003)

Various sugars (glucose, fructose, lactose, mannose and sucrose), sugar alcohols (sorbitol and inositol) and non-reducing sugars (trehalose) were found to be the effective protection (Carvalho *et al*., 2004). Morgan *et al.* (2006) reported that

sugars such as trehalose and sucrose enhanced desiccation tolerance in numerous organisms because these sugars replaced the water around polar residues within the macromolecular structures. The stabilization of membranes and proteins were also occurred. In addition, these two disaccharides preserve structure and function of isolated proteins during drying by the formation of hydrogen bonds which help to maintain the tertiary protein structure in the absence of water. Trehalose was found to be a potential protectant for freeze-dried *L. acidophilus* (Conrad *et al*., 2000). Likewise, trehalose greatly enhanced survival of *L. salivarius* when used alone or together with other protective materials. It was found that the skim milk with addition of trehalose and sucrose were the most efficient materials with survival rate of 83–85% immediately after freeze-drying (Zayed and Roos, 2004). The protective effects of disaccharides on *L. rhamnosus* GG survival during freeze-drying were investigated by Meng *et al.* (2006). The results demonstrated that trehalose, trehalose/lactose and lactose/maltose were the most efficacious disaccharides during both freezing and freeze-drying. Sucrose also has quite frequently been used for the cryopreservation of various microorganisms such as *Lactococcus lactis* sp. lactis, *L. delbrueckii*, *Methanococcus vannielii*, *Chlamydia* sp., *Mycoplasma* sp. (Hubálek, 2003).

The ability of monosodium glutamate (MSG) to protect viability and activity of distinct microorganisms during cryopreservation and freeze-drying was described by a number of researchers (Hubalek, 1996; Martos *et al*., 1999). Abadias *et al.* (2001) claimed that MSG was very effective in preserving *Candida sake* cells during lyophilization. In contrast, Carvalho *et al.* (2002) reported that no significant differences were observed in the viability of *L. plantarum* and *L. rhamnosus* cells during freeze-drying in the presence or absence of monosodium glutamate. Similarly, the addition of MSG or sorbital did not improve the survival of *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *E. durans* and *E. faecalis* after freeze drying. However, these compounds increased the stability of strains during long-term storage (Carvalho *et al*., 2003).

2.2 Spray drying

Spray drying is a predominant process for drying microorganisms. It is an inexpensive process, easy to scale up, high yields and high production rates comparing to freeze drying process (Anal and Singh, 2007; Gardiner *et al*., 2000; Morgan *et al*, 2006). In this process, the starting material is in liquid or paste. The slurry from a nozzle is atomized into a high flow velocity of hot air (up to 200 °C) and it is then dried into granules before they hit the side of the chamber (Morgan *et al*., 2006). Consequently, this process results in exposure of the drying medium to high temperature for a short time which can be detrimental to the integrity of live bacterial cells (Ananta *et al*., 2005). Moreover, it was found that the outlet air temperature (the temperature at which the product leaves the drying chamber) is the critical parameter affecting viability of spray dried probiotic cultures (Desmond *et al*,. 2002). The other factors which had affected on the stresses of bacterial was also reported including oxidative, dehydration-related stresses (osmotic, accumulation of toxic compounds, etc.) acting either simultaneously or successively on bacteria, which potentially lead to cell death (Ananta, 2005). Besides, the removal of water, which contributes to the stability of biological molecules, may cause irreversible changes in the structural and functional integrity of bacterial membranes and proteins. Teixeira *et al*. (1995) revealed that the effect of spray-drying on the cell membrane can lead to increase cell permeability which may result in the leakage of intracellular components from the cell into the surrounding environment. The stress of cytoplasmic membrane is the most susceptible sites in bacterial associated with spray drying. In addition, the cell wall, DNA and RNA are also loss metabolic activity (Teixeira *et al*., 1997). However, the addition of protective agents to the media prior to drying such as sugar (e.g. trehalose, sucrose, lactose, glucose), skim milk powder, whey protein, monosodium glutamate (MSG), polymer (e.g. dextran polyethylene glycol) and starch have been showed to improve probiotic viability during drying (Lian *et a.l*, 2002; Morgan *et al*., 2006, Anal and Singh, 2007; Meng *et al*, 2008).

The successful spray drying of probiotic has been reported for a number of different strains, including *L. paracasei*, *L. curvatus*, *L. acidophilus*, *L. rhamnosus* and

B. ruminantium (Anal and Singh, 2007). However, the successful of spray- dried probiotic as the high cell viability after finished process depends on many factors such as the probiotic strain, outlet temperature, and drying medium (Meng *et al*., 2008). Lately, the survival of various bifidobacteria strains after spray-drying with different carrier media including 10% (w/w) gelatin, gum arabic, soluble starch and skim milk was investigated. It was found that *B. longum* B6 exhibited the least sensitivity to spray-drying and showed the highest survival of ca. 82.6% after drying with skim milk (Lian *et al.*, 2002). Similarly, the viable bifidobateria cell number of 10^8cfu/g after spray drying was obtained by using skim milk as the protective media (Simpson *et al*., 2005). The addition of reconstituted skim milk (20% w/v) also gave the high survival rate (84.5%) of *L. paracasei* NFBC 338 (Gardiner *et al*., 2002). Furthermore, the incorporation of reconstituted skim milk with gum acacia in medium prior to spray drying enhanced the 10-fold viability of *L. paracasei* NFBC 338 (Desmond *et al*., 2002). Besides, Hamsupo (2005) claimed that the viable cell of *L. reuteri* KUB-AC5 was slightly decreased in presence of skim milk as drying medium. In addition, skim milk was also recommended for spray drying of *L. rhamnosus* GG (Ananta *et al*., 2005). Recently, the use of prebiotics such as inulin or polydextrose as carrier media to enhance probiotic survival during processing spray-drying are interesting but it was found that they did not enhance viability of *L. rhamnosus* GG during spray-drying (Corcoran *et al*., 2004). On the other hand, survival of *L. sakei* CTC 494 was improved by the presence of sucrose and monosodium glutamate into the medium (Ferreira *el al*., 2005).

3. Method of administration of probiotic in poultry

The administration of probiotic in poultry has four difference methods such as (1) treatment of individual chicken, (2) administration via drinking water, (3) droplet and spray application and (4) administering through the feed (Gradban, 2002).

(1) Treatment of individual chicken

Practically, there are four difference ways for treating poultry individually including introducing the treatment material into the crop by tube and syringe or into the beak using a hypodermic syringe fitted with a beaded needle, allowing each chick to drink from the tip of a pipette and dipping the beak of the bird in the treatment material. In laboratory trials, the intubation into the crop and administration via the beak is also commonly used and the beak dipping may be appropriate in some circumstances (Gradban, 2002). However, Mead *et al*. (1989) recommended the method which allows chicks to drink from the tip of pipette.

(2) Administration via drinking water

Administration of probiotic via drinking water was the first method which used for commercial and this approach was used for many years in Finland and Sweden. However, this method has several disadvantages. Mead (2000) described that sometimes some of the chicks refused to drink or failed to drink before feeding and probiotic preparation may spread unevenly among the flock. In addition, probiotics was likely to die during process due to the oxygen toxicity. Perhaps the main disadvantage of using drinking water as a vehicle for the treatment of poultry is the inevitable delay between hatching and placement of broiler in the rearing houses. For this reason, methods of administration have been sought that would allow probiotic treatment to be applied at the earliest point in production.

(3) Spray application

A method of spray application was developed by Goren *et al*. (1984) and chicks were treated either in the delivery boxes, just prior to leaving the hatchery, or in the hatchers themselves. Wolfenden *et al.* (2008) reported application of probiotics to large numbers of chicks should be administered as early in life as possible and should minimize uncontrolled variables such as water quality and medicator function and consistency. These issues can be minimized if the probiotic was administered at the

hatchery by this method. Furthermore, spraying in the hatchers with subsequent administration in drinking water on the farm was described by Blankenship (1993). This appears to have been a precaution to ensure that all broiler received adequate treatment (Wolfenden *et al.*, 2008) and this method gave highly effective for the control of Salmonellae. Ghadban (2002) reported that Ghadban (1999) used spray application and followed by treatment of the chicks through their first drinking water to controlling the pathogenic bacteria. The result showed that this method was a highly effective in controlling *Salmonellae* and *E. coli* and improving growth performance of chickens.

(4) Administering through the feed

Mixing the probiotic with the feed ingredients is a method that could be used for application to poultry. Timmerman *et al*. (2006) reported that administration of probiotics via the feed generally resulted in a higher increase of average daily gain compared to administration in the drinking water. However, the incorporation of probiotics like Lactobacillus or Streptococcus into the pelleted feed rarely produces. It is most likely due to the fact that the lactic acid bacteria are destroyed by pelleting process which use high temperature (Gradban, 2002).

4. Development of pelleted feed containing probiotic

In recently decade, use of poultry feed in the form of pellet is the most popular because it has a lot of benefits such as decreased feed wastage, reduced selective feeding, decreased ingredient segregation, less energy spent for prehension, destruction of pathogenic organisms, improved palatability (Behnke, 1994; Pesti *et al*, 2005) increasing the bulk density of feed, improving feed flowability and providing opportunities to reduce feed formula costs through the use of alternative feed ingredients, easy for handling. Moreover, it was found that feed in form of pellet also provided the other positive effects on the poultry performance for instance improved body weight gain, improved feed conversion ratio and increased digestibility (Fairfield, 2003). Jahan *et al.* (2006) reported that mash diet gives greater unification

of growth, less death loss and more economical, but ground feed is not so palatable and does not retain their nutritive value.

A number of studies have been reported the performance of broiler when chicken fed difference feed form. It was found that the difference of form between pellet and mash did not affect on feed conversion and mortality of broiler. However, the use of pellet form was able to increase broiler body weight and carcass yields (Jones *et al*., 1995). According to Plavnik (1997), the growth responses of broilers and turkeys to pelleted diets were higher than mash diets and feeding of pelleted diets resulted in an increase in abdominal fat of both broilers and turkeys. Similarly, Rincon and Leeson (2002) discovered that the broiler fed mash diets had the body weights and the breast meat yields lower than broiler fed pellet diet. In addition, the reducing of cumulative mortality of broilers was shown in broilers fed pelleted diets. Recently, the effect of feeding different forms of feed (mash, pellet and crumble) on the productive performance of broiler was investigated. The result represented that the highest, intermediate and the lowest body weight of broiler were obtained from crumble, pellet and mash group, respectively. Moreover, the feed form affecting on animal performance depended on age of broiler (Jahan *et al.,* 2006). According to Salari *et al.* (2006), chickens fed pelleted diets consumed more feeds and had weight gain, weight of abdominal fat and feed conversion ratio higher than mash diets group. Furthermore, it was also found that a poor feed form could inhibit feed intake and had a negative impact on growth rate of chicken and the lowest broiler feed intake was obtained by using the mash diet (Lemme *et al*., 2006). In addition, the improvement in chicken performance with pellet was accompanied by a decrease in the relative length of all components of the digestive tract. Conversely, the extent of the mucosal layer was greater in both the duodenum and jejunum of broiler fed pelleted feeds (Amerah *et al*., 2007).

4.1 Poultry pelleted feed manufacturing

Feed manufacturing involves the use of variety raw materials to produce compound feeds and includes a wide range of unit operations such as receiving,

grinding, mixing, conditioning, pelleting, drying/cooling, loadout and delivery. The general plan of feed processing was represented in Figure 1.

(1) Ingredient receiving

Before uploading, samples of all ingredients should be taken from each delivery and assayed for nutritional properties and potentially harmful substances. If they contain any harmful substances, they could contaminate anything still in the bin. Especially, cereals should be checked for moisture, mold growth, unusual odors or colors, rodent pellets and other foreign material. The moisture level of ingredients such as corn, barley, rice and sorghum should control at below 13% and below 12% for soybeans in order to prevent contaminating during storage. The fungal growth should be near zero but even with low moisture and temperature, some fungal will occur if ingredients or feed are stored too long. Moreover, if there is free oxygen present, oxidation of some vitamins will also occur with time. Aeration fans in larges bins help reduce temperature and moisture level and keep them uniform throughout the bin and reducing spoilage (Pesti *et al*., 2005).

(2) Grinding or milling

Grinding is a major function of feed manufacturing and is the most common method of feed processing. Grain is ground prior to mixing for increasing surface area which is easy to get a homogenous mixture (Pesti *et al*., 2005), decrease segregation, improved mixing problems, increase rate of digestion and facilitate future processed such as extrusion or pelleting (Behnke, 1996). Grinding is accomplished by many types of manual and mechanical operations involving impact, attrition and cutting. Of the various mill designs that can be used to grind feed stuffs, roller mill and hammer mill are the most commonly used in the production of animal feed. During grinding, the increasing of shrink through moisture and dust losing will occur. In addition, some starch gelatinization may occur and the ability to gelatinize for making pellets later can be lost, if the temperature gets too high (Pesti *et al*., 2005).

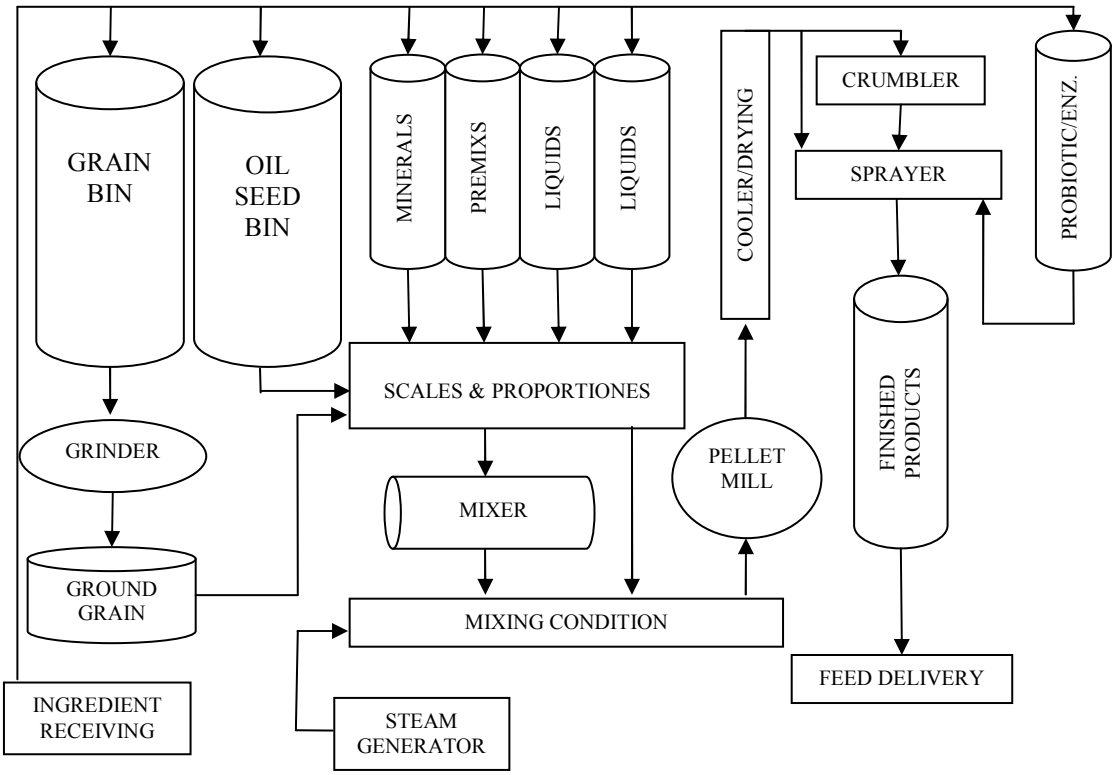


Figure 1 General plan of feed processing

Source: Modified from Pesti *et al.* (2005)

(3) Mixing

Mixing is an operation basic to feed manufacturing. When ingredients are combined to be fed as a complete diet, they must undergo a mixing process and intuitively nutrient uniformity in a complete site is necessary to maximize nutrient utilization (Engelen and Van der poel, 1999).

(4) Conditioning

Conditioning in animal feed manufacturing can be defined as the process of converting the mixed mash with the use of heat, water, pressure and time to a physical state that facilitates compaction of the feed mash (Thomas *et al*., 1997). For this process, the conditioner most widely used to incorporate water, steam and/or other

liquids (above 82°C) which was supplied for gelatinization (Pesti *et al*., 2005) or alternation components like starch and protein in the feed mash, in a way that binding properties come into effect (Thomas and van der Poel, 1996). During condensation of the steam, a thin film of water is created around the particles, which, together with the temperature increase, facilitates binding between particles. The effects of steam addition include the water itself may exhibit bonds via capillary sorption between particles and the heat and water induces a wide range of physical and chemical changes, including among others, thermal softening of the feed, denaturation of proteins and gelatinization of starch which is normally enhanced when some form of residence time is incorporated. In addition, applying too much heat or water, however, will impair production capacity and pellet quality and may lead to plugging of the pellet press (Thomas *et al*., 1997). The product when leaves the conditioner it is typically 82-90°C (Pesti *et al*., 2005). Moreover, the application of steam increases the moisture content (to 15-18%) and heat of the feed mash because steam is more homogeneously dispersed through the feed mash.

(5) Pelleting

Pelleting is the process of forming a mash feed into larger, homogenous particles. The feed is agglomerated by mechanically compacting and it through die openings. After pelleting, the feed mash was formed to the cylindrical particles that have a uniform diameter, usually between 2.5 and 5.0 mm. for poultry and the temperature of pellet was typically 82-90°C, while the pellet moisture was 15-18% (Pesti *et al*., 2005). The pelleting process was originated from almost over 60 years ago. The early pelleting process involved mixing the feed ingredients and pelleting them by pressing process with no further treatment. The mash was simply pressed between two rollers or in a cake press to obtain pellets/cakes by low pressures and the feed did not heat up (Thomas and van del Poel, 1996). The rationale for this approach was to prevent alterations to vitamins and proteins due to the addition of heat to the feed mix (Gao *et al*., 1999). In the late 1930's the modern feed mill was utilized, it was called roller and die pellet press both vertically and horizontally. Before entering the pellet press, the mash is subjected to some form of pretreatment before granulation

such as mixing with molasses or conditioning with steam or being subjected to the use of an expander to increase temperature or moisture level (Thomas and van del Poel, 1996). Pesit *et al*. (2005) claimed that when the feed leaves the expander, it is typically 100-110°C and contains 17-18% moisture. Expanders could also be called high temperature short time conditioners and angular gap expanders. They provide hydrothermal pressure temperature of the feed after the conditioner and before the pellet mill. Water and steam may be added to the expander barrel with the raw material which is transported down the barrel. As the raw materials pass further down the barrel of the expander, temperature, pressure and internal shear forces rise. The combinations of different phenomena, densifying the feed mash, shearing and mixing will affected on the physicochemical properties of the feed mash and alter the structure to enhance the binding between feed particles in the pelleting process which leads to increase durability (Thomas *et al*., 1997).

(6) Drying

After pellet leaves from the die, its moisture content was ranking of 15% to 18% which is suitable for undesirable microorganism growth. Thus, the drying process is included in feed manufacturing in order to decrease moisture for prevention and reproduction of the microbial growth. In addition, the drying process provided opportunities to increase quality by maintaining the structural integrity of the pellet, reduced in product weight and volume, decreased packing, storage and transportation costs and enhanced flow properties (Thomas *et al*., 1997). Pellet drying process is accomplished by many types of convective dryer such as belt conveyer dryer, fluid bed dryer, rotary dryer, tray dryer and conduction dryers such as steam jacket rotary dryer and steam tube rotary dryer (Mujumdar, 1995).

(7) Crumbing

Especially for very young chicks, even 2.5 mm. pellets are too big and their size must be reduced. This is accomplished by passing the pellets between two grooved rollers in roller mill (Pesti *et al*., 2005).

(8) Spraying

Some supplemental enzymes and probiotics are less heat stable. They should be sprayed on pellet after the heat treatment for maintained their activity (Pesti *et al*., 2005).

4.2 Pelleted feed containing probiotics manufacturing

Probiotics are incorporated into animal feed as specific additives in different ways (during or after processing or top-dressing feed mix) but the addition of probiotic in the pelleted feed which usually fed to broiler is rarely produce. It is probably because feed manufacture involves pelleting, extrusion process and complementary processes, requires pressures and high temperatures (65-85 °C). This harsh condition may militate against the viability of probiotics that applied in the feed (Fajt, 1996; Engelen and van der Poel, 1999; Gao *et al*, 1999; Aguirre-Guzmán *et al*., 2002). For example, Aguirre-Guzmán *et al*. (2002) reported that the survival of 0.5% and 1.0% *S. cerevisiae* in shrimp diets after extruding in a meat grinder (72°C for 30 s) and drying in an air-forced oven (55°C for 15 h) was only 0.62% and 0.02%, respectively. Moreover, they found that the viable level of *S. cerevisiae* in the shrimp pelleted feed produced by commercial shrimp pellet mill at 82°C for 12 seconds decreased from 10^7 to 10^2cfu/g. *Bacillus* which is well known for being able to produce spore and survive at high temperatures has been recommended for adding to the feed before pelleting. Simon (2005) claimed that the recovery of 95% *B. cereus* toyoi was obtained after pelleting at 87°C. *B. subtilis* PB6 was inoculated into the mash feed which was exposed to conditioner various temperatures set (70-90°C) prior to pelleting. It was found that a minimum viable cell of 1 log cfu/g will be lost when hot pellet temperatures reach greater than 90°C (Lundeen, 2008).

Furthermore, the cold process is possible technology to produce pelleted feed containing probiotics. The process has been established by Gao *et al.* (1999). It is different from the current pelleting process by eliminating the thermal process as conditioning step involving the use of steam and elevated temperature. The liquid

binder as Brewex (a concentrated molasses-like by-product of the brewing industry), corn streep liquor, sugar syrup and condensed liquid whey or combination organic binder (pregelatinized starch, CMC, alginate ester, guar gum and collagen) with fresh water (Fajt, 1996) are used in place of steam and pelleted at ambient temperature. The pellets obtained from this process give typical 37°C-65°C depending upon the diet formula, type of binders and level of binder used. Cold pelleting process consists of 5 steps including batching, mixing, pelleting, drying/cooling and screening. After batching, the dry ingredients are mixed in the mixer. Then the liquid ingredients as fat are added and mixed. Liquid binder is added last by blending the binder into the mix to obtain a uniform cohensive mash. Then, the mash is delivered to the pellet mill and pelleted at ambient temperature. When the mash is compacted through the die, soft moist pellets are formed. The pellets had a temperature below 65°C and a moisture content of 16-17%. The wet pellets were dried in a cooler or dryer to obtain moisture content below 12%. The durability index (PDI) may range from 55-60 for corn soy diets and 85-90 for wheat-based diets (Gao *et al.,* 1999). According to Daigle *et al*. (1997), cold extrusion was used to make fungal biocontrol preparations in a refrigerated twin screw at 25°C followed by fluidized bed drying (50°C, final moisture 4-7%). It was found that survival of 0.1-5.3% for *Collectotrichum truncatum*, 0.9-83% for *Alternaria* sp., 16.7-91.05% for *Paecilomyces fumorosoceus* and 86-100% for *Aspergillus* sp. were obtained.

5. Survival of probiotics during storage

The storage condition will influence the shelf life of the probiotic products and the suitable conditions are essential to maintain viable populations of probiotics at sufficiently high levels to assure their therapeutic activity throughout shelf life. The effect of storage condition has been studied by many researchers. It was demonstrated that oxygen, moisture, light, microbial contamination and elevated temperatures can greatly affect the viability and stability of probiotic during storage (Carvalho *et al*., 2004; Morgan *et al*., 2006; Chávez and Ledeboer, 2007; Meng *et al*., 2008; Santivarangkna *et al*., 2008).

Temperature is a critical parameter which is inversely related to cell survival (Teixeira *et al*., 1995; Selmer-Olsen *et al*., 1999; Carvalho *et al*., 2002). Gardiner *et al*. (2002) demonstrated that the viable cell of spray-dried *L. paracasei* NFBC 338 with reconstituted skim milk was quite stable during storage at 4°C and 15°C whereas, the viable of the strain declined from 10^8 to 10^6cfu/g during storage at 30°C for 49 days. In agreement with the results published by Desmond *et al.* (2002), the survival of the strain in gum acacia containing powders was highest during storage at 4°C, while the decline rate of viable cell increased when powders were stored at 15°C and 30°C for 8 weeks. Moreover, the spray-dried *L. reuteri* KUB-AC5 showed high viable cell during storage at 4°C for 4 months, but the total number of the strain decreased as the storage temperature increased to 30°C (Hamsupo *et al*., 2005). Similarly, Simpson *et al*. (2005) reported that spray-dried *Bifidobacterium* sp. had high survival during prolonged storage at 4°C, while a significant decline of the strains were observed when storage at 15°C and 25°C. Furthermore, the viable cell of *L. plantarum* 44a in shrimp feed was quite stable during storage in refrigeration for 1 year. The viable strain was maintained in refrigeration probably because bacteria metabolism decreases (Bucio *et al*., 2005) and the accumulation of toxic wastes from the metabolism was likely minimized (James *et al*., 2007).

In view of the ecology of the strains normally used in probiotic products, the anaerobic or microaerophilic bacteria, the low oxygen content has been cited as important factors for the viability of probiotic during storage (Talwalkar and Kailasapathy, 2004). According to da Cruz *et al*. (2007), the level of oxygen in the probiotic during storage should be as low as possible. It is most likely because probiotic bacteria lack effective oxygen scavenging cellular mechanisms such as catalases (an enzyme essential to the breakdown hydrogen peroxide which was by-product of oxygen respiration mechanism). Hence, exposure to oxygen in these bacteria causes toxic oxygenic metabolites to accumulate in the cell leading to cell death from oxidative damage (Talwalkar and Kailasapathy, 2004). Moreover, it may lead to loss of membranes function, block DNA replication and cause mutations (Cabiscol *et al*., 2003). For this reason, probiotic products should be practically contained in package which is able to prevent oxygen permeation. Wang *et al*. (2004)

monitored viable cell counts of *Streptococcus thermophilus* and *Bifidobacterium bifidum* in fermented soy milk filled into glass packages, polyethylene packages containing an oxygen absorber and a desiccant, and laminated bag (nylon/aluminum/polypropylene) and stored at 4°C. The laminated material exhibited the best performance, with a reduction of only 0.55 log cfu/g and 29.5% survival rate after 4 months. In addition, Champagne *et al.* (1991) revealed that vacuum storage was found to be better than nitrogen and air, respectively. According to Morgan *et al.* (2006) and Buio *et al*. (2007), the viability of probiotic during storage under vacuum or nitrogen gas was superior compared to storage in air.

The protective agents used during drying of probiotics are also known to enhance storage viability and stability. However, the efficiency of these compounds may exert different due to difference in chemical characteristics and physical properties (Lian *et al*., 2002). Moreover, the degree of protection during storage afforded by a given additive is species and strain dependent (Carvalho *et al*., 2003; Morgan *et al*., 2006). The protective effects of sucrose, fructo-oligosaccharide and inulin on viability freeze-dried *L. reuteri* TMW1.106 were similar or better than the effects of skim milk during storage at room temperature in the dark for 14 days (Schwab *et al*., 2007). In contrast, sucrose did not preserve viability of freeze-dried *L. salivarius* during storage at −85°C for 7 weeks. However, the use of trehalose showed the survival rate higher than skim milk (Zayed and Roos, 2004). Moreover, various sugars have been tested for their protective effect on survival of freeze-dried *L. plantarum*. It was found that sorbitol was the most effective protectant during storage, while the least effective protective was obtained by using trahalose during storage at 20°C for 10 months (Carvalho *et al*., 2002). After storage at 4^{o}C and 30^{o}C for 3 months, the spray dried *L. reuteri* KUB-AC5 in presence of monosodium glutamate (MSG) gave the highest survival compared to ascorbic acid and soluble starch both (Hamsupo, 2005). According to Carvalho *et al*. (2003), the addition of sorbitol and MSG to the skim milk as drying medium increased survival of *L. bulgaricus* and *L. rhamnosus* during storage. Ananta *et al*. (2005) evaluated the effect of a spray-dried protective agent on protection of *L. rhamnosus* GG at 25°C and 37°C storage conditions, and found that the protection capacity decreased in the order reconstituted

skim milk (RSM) > RSM/Polydextrose > RSM/Raftilose. Similar results were reported by Corcoran *et al.* (2004). *L. rhamnosus* GG when spray-dried in RSM in the stationary phases and stored at 37°C for 8 weeks retained good viability. Furthermore, spray-dried *L. paracasei* was grown in a mixture of reconstituted skim milk and gum acacia had survived better than untreated cultures during storage at 4–30°C for 4 weeks (Desmond *et al*., 2002).

MATERIALS AND METHODS

Materials

1. Microorganisms

Lactobacillus reuteri KUB-AC5, isolated from the chicken intestines (Nitisinprasert *et al*., 2000) was obtained from the collection of the Department of Biotechnology, Faculty of Agro-industry, Kasetsart University, Thailand. The strain was preserved in MRS broth (Merck, Darmstaat, Germany) containing 20% (v/v) glycerol at –80°C. The culture was propagated twice in MRS broth prior to use as the inoculum for the experiments.

2. Chemicals and media

2.1 Agar (Britania, Argentina)
2.2 Calcium carbonate ($CaCO_3$; Univar, Ajak, NSW, Australia)
2.3 Carboxymethlycellulose (high viscosity) (Fluka, United Kingdom)
2.4 Glycerol (Carlo Erba reagent, U.S.A.)
2.5 Modified starch (Commercial grade)
2.6 Monosodium glutamate (Commercial grade)
2.7 MRS broth and agar (Merck, Germany)
2.8 Nutrient broth (Himedia, India)
2.9 Sodium alginate (Fluka, United Kingdom)
2.10 Sodium chloride (NaCl; Univar, Ajak, Australia)

3. Apparatus

3.1 Autoclave (Tomy, Model ES-315 , Japan)
3.2 Balance 2 digit (Sartorius, Model ED32023, Germany)
3.3 Balance 4 digit (Ohaus, NJ, USA.)
3.4 Centrifuge (Hermle, Germany)

3.5 Hardness tester (Lloyd, Model LRX plus, England)

3.6 Hand Mixer (Moller, China)

3.7 Hot air oven (Memmert, Model UNE 200, Germany)

3.8 Incubator (Memmert, Model INE 200, Germany)

3.9 Laboratory pellet mill (California pellet mill, USA.)

3.11 Spray dryer (Niro, A/s, Denmark)

3.12 Vortex mixer (Genie, Scientific Industries, USA)

Methods

1. Preparation of *L. reuteri* KUB-AC5

L. reuteri KUB-AC5 were subcultured at 5% in MRS both at 37°C for 24 h. They were then inoculated into 500 ml MRS broth and incubated at 37°C until stationary phased was reached (Hamsupo, 2005).

2. Preparation of spray-dried *L. reuteri* KUB-AC5

The cell suspensions of *L. reuteri* KUB AC5 were mixed with 20% (w/v) milk powder and directly spray dried. The constant inlet air temperature and outlet air temperature was 130°C and 70°C respectively (Hamsupo, 2005). Dried samples were collected from the cyclone and counted viable cell.

3. Determination of drying time for dying pelleted feed containing *L. reuteri* KUB-AC5 at 40°C and 50°C

Dry feed ingredients (Table 3) were ground and sterilized at 121°C for 15 minutes. Then, they were dried in hot-air oven at 55°C until 12-13% moisture content was reached (Pesti *et al*., 2005). Dry feed ingredients and modified starch (1%, 2% and 3%w/w) were mixed by handle mixer and water (20%, 25% and 30% v/w) was added to the diet. Immediately after water addition, the mash diets were mixed for 3 min followed by soybean oil addition then mixed for 3 min again. Subsequently, the

mash was conveyed to the laboratory pellet mill and pelleted at ambient temperature. The wet pellets of each treatment were divided into two groups for drying at 40°C and 50°C. The pellets were collected at time interval to determine moisture content.

Table 3 Composition of broiler diet offered to 22-42 days broiler

Composition ingredients	Weight (%w/w)
Yellow corn	66.19
Soybean meal	23.26
Meat meal	8.00
Soybean oil	1.00
Limestone	0.50
Calcium phosphate (P/18)	0.25
Salt	0.30
Premix	0.50

Source: NRC (1994)

4. Production of pelleted feed containing of *L. reuteri* KUB-AC5

4.1 Viability of *L. reuteri* KUB-AC5 during drying process

The cultures of *L. reuteri* KUB-AC5 (20%, 25% and 30% v/w) and modified starch (1%, 2% and 3% w/w) were added into the diets. The mash with *L. reuteri* KUB-AC5 was conveyed to the pellet mill and pelleted at ambient temperature. After that, the wet pelleted were dried by hot-air oven at either 40°C or 50°C until the moisture content was lower than 11%. During drying process, the pelleted were collected to evaluate viability of the strain.

4.2 Effect of protective agents on viability of *L. reuteri* KUB-AC5 in pellet during drying process

The cell suspension was mixed with protective agents at different contents (Table 4). Then 25% (v/w) of mixture and 2% of modified starch and diet ingredients were mixed. The mash with *L. reuteri* KUB-AC5 was fed into pellet mill followed by drying at either 40°C or 50°C until the moisture content was lower than 11%. During drying, the pellets with *L. reuteri* KUB-AC5 were collected to investigate the viable cell of *L. reuteri* KUB-AC5.

Table 4 Amount of protective agents used for production pelleted feed containing *L. reuteri* KUB-AC5 with protective agents

Protective agents	%w of protective agent/v of fresh liquid culture
Carboxymethyl cellulose (CMC)	1.5
Sodium alginate	3
Milk powder	20
Monosodium glutamate (MSG)	10

4.3 Effect of modified starch and protective agents on pellet hardness

The pelleted feed containing *L. reuteri* KUB-AC5 of 4.1 and 4.2 was measured the pellet hardness by hardness tester. Ten replicates were done for each treatment.

4.4 Viability of spray-dried *L. reuteri* KUB-AC5 during drying process

1% of spray dried *L. reuteri* KUB-AC5, dry ingredients, modified starch (1%, 2% and 3% w/w) and water (25% w/v) were mixed. The mash was fed into the laboratory pellet mill. Then, the wet pellets with the strain were dried in hot-air oven at 40°C and 50°C until was close commercial feed moisture content. During drying, the pellets with the strain were collected to investigate the viable cell.

The overall procedures were summarized as shown in the following diagram.

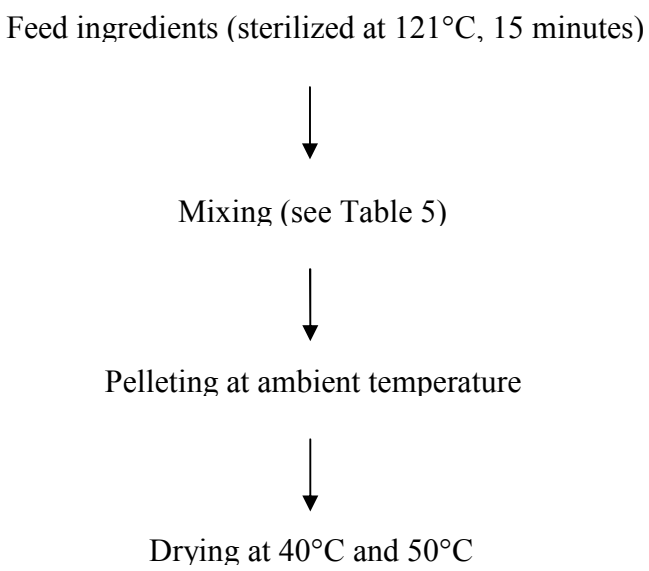


Table 5 Composition of ingredients using for mixing process

Experiments		Composition of ingredients			
		L. reuteri KUB-AC5 (%v/w)	Modified starch (%w/w)	Water (%v/w)	Protective agents
Production of pelleted feed containing *L. reuteri* KUB-AC5					
I.	Without protective agent	20, 25, 30	1, 2, 3	x	x
II.	With protective agent	25	2	x	see Table 4
III.	Using spray-dried *L. reuteri* KUB-AC5	1%(w/w) spray-died culture	1, 2, 3	25	x

5. Stability of *L. reuteri* KUB-AC5 in pelleted feed during storage

The pelleted feed with *L. reuteri* KUB-AC5 of 5.1, 5.2 and 5.4 were stored in sealed aluminum bag. The samples were kept at 4°C and 30°C for period of 150 days. The viable cells of the strain were determined at time intervals to investigate their stability.

6. Analytical procedures

6.1 Viable cell numbers

The viable cell number of *L. reuteri* KUB-AC5 was counted on a MRS agar plate with 0.4% $CaCO_3$. The pelleted feed with *L. reuteri* KUB-AC5 was blended by stomacher and serially diluted (1:10) in 0.85% NaCl solution and plate counted on 1.5% MRS agar, duplicate plates were done for each dilution. Then, the MRS plates were incubated at 37°C and the plates were enumerated for colony forming units per milliliter (cfu/ml) after 24 h. Duplicate was done for each treatment. The percentage survival was calculated as followed:

$$\% \text{ survival} = {}^{N}/_{N_0} \times 100$$

Where N_0 and N represent the number of *L. reuteri* KUB-AC5 in pelleted feed before and after drying/storage, respectively.

6.2 Moisture content of broiler pellet

Moisture content was determined by an aliquot of about 1 g in a moisture can and was dried it in an oven at 105°C until the weight was constant. The results were expressed in percentage of the moisture.

7. Place and duration

Department of Biotechnology, Faculty of Agro-industry, Kasetsart University, Thailand

The experiments were carried out from October 2006 to January 2009.

RESULTS AND DISCUSSION

1. Determination of drying time for drying pelleted feed containing *L. reuteri* KUB-AC5 at 40°C and 50°C

In order to decrease moisture content of pelleted feed to lower than 11% (Uppal *et al.,* 2002), the pellet was dried at 40°C and 50°C with hot-air ventilation. The initial moisture content of the pellet was ranking of 23-30%. The drying curves at various conditions were shown in Figures 2 and 3. They were plotted between moisture content and drying time. At both drying temperature, all of samples showed the characteristic sigmoidal drying curve typically seen for materials subjected to dehydration (Porter *et al*., 1997). The moisture content of all samples decreased gradually as time passed.

Figure 2 showed that the moisture content decreased dramatically from ca. 23% to 12% after 6 h drying at 40°C and it reached lower than 10% after 8 h., while this moisture content was obtained when dried at 50°C for 4 h (Figure 3). Thus the optimum drying time of 40°C and 50°C was 8 h and 4 h, respectively. Comparison of Figure 2 and 3, dehydration of pelleted feed at 50°C was faster than that at 40°C. The results were supported by Methakhup, (2003). Drying at higher temperature gives shorter time to reach the equilibrium moisture content than drying at lower temperatures. Similarly, Bayrock and Ingledew (1997) reported higher temperature dehydrated the baker's yeast faster than lower drying temperature.

Moreover, the results showed that the content of modified starch had a little effect on the drying rate. 1% of modified starch content exhibited the fastest rate in both conditions of drying. However, there was no significantly different ($P<0.05$). The result was supported by Ruscoe *et al*. (2005); the drying rate was decreased with increasing binder content. The starch granules are accumulations of numerous starch molecules that can be fractionated into essentially linear chain amylose and the highly branched amylopectin. Water absorption ability, stickiness and hardness are affected by the amylose content. The amylose chain displays a natural twist giving a helical

conformation. Hydroxyl groups of glucosyl residues were located on the outer surface of the helix. When the water was added, the binding between hydroxyl group of amylose and hydrogen atom of water were created (Zhou *et al*., 2002). The free water in the solid also decreased. Therefore, the increasing of modified starch content will affect the rate of drying to be slower.

2. Production of pelleted feed containing of *L. reuteri* KUB-AC5

The objective of this work was to develop a probiotic pelleted feed. Pelleting process was preceded by low temperature and followed by drying at 40°C for 8 h and 50°C for 4 h in order to decrease moisture content in the pellet (lower than 11%) and to prevent deterioration over several months. However, it will affect the survival of probiotic culture.

2.1 Production of pelleted feed containing *L. reuteri* KUB-AC5 without protective agent

2.1.1 Viability of *L. reuteri* KUB-AC5 in pelleted feed without protective agents during drying process

The viability of *L. reuteri* KUB-AC5 in pelleted feed during drying at 40°C and 50°C were shown in Figure 4 and 5, respectively. It was represented by the logarithmic values. The loss of viable cell was gradually increased as the drying time elapsed. After drying at 40°C, the viable cell in all treatments was decreased approximately 1 log cycle (from ca. 10^8-10^9cfu/g) as shown in Figure 4. While drying at 50°C, the remained viable cell was slightly lower and the reduction was approximately 1-2 log cycles as displayed in Figure 5.

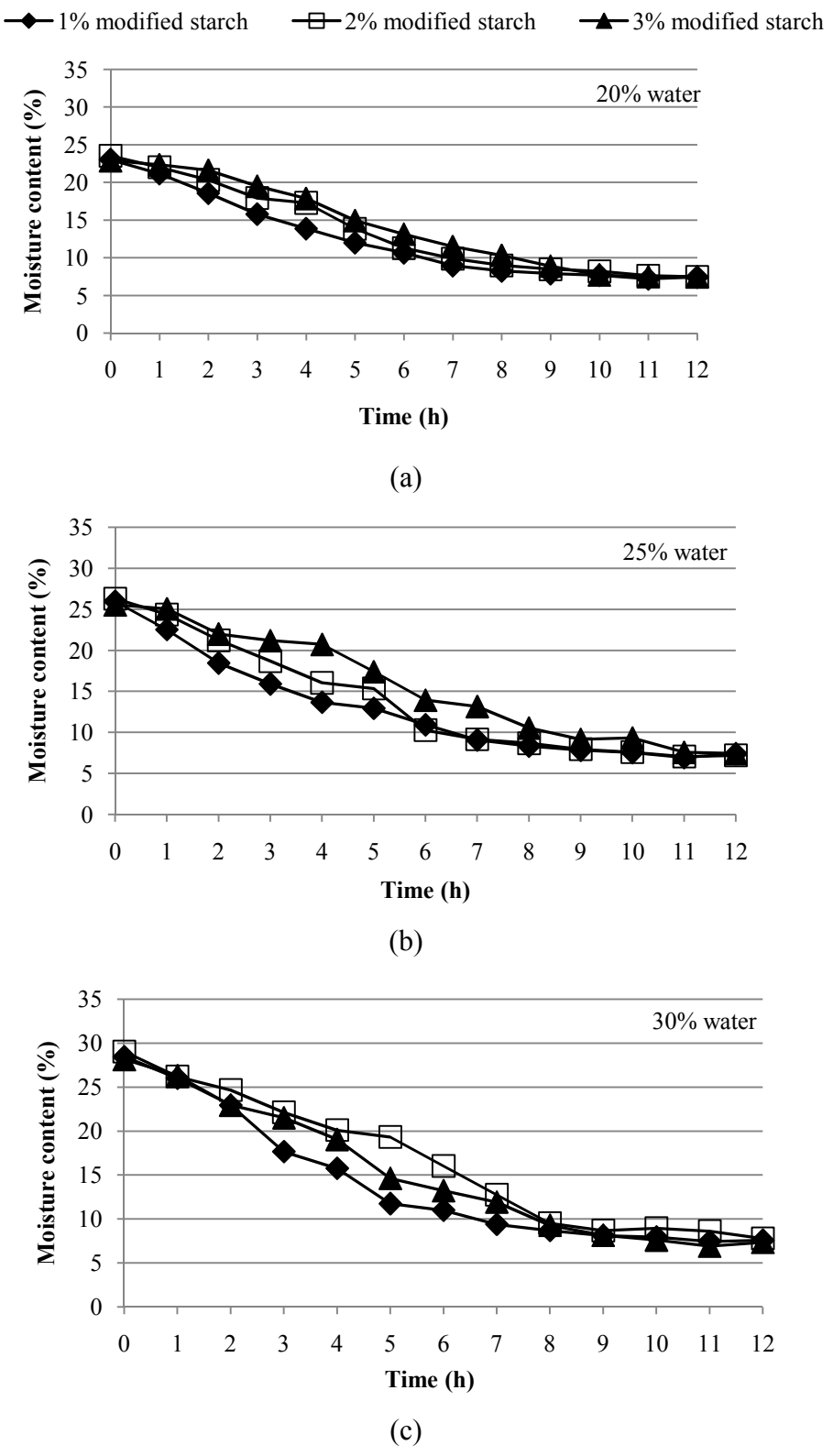


Figure 2 Drying curves of pelleted feed with various treatments undergoing drying at 40°C

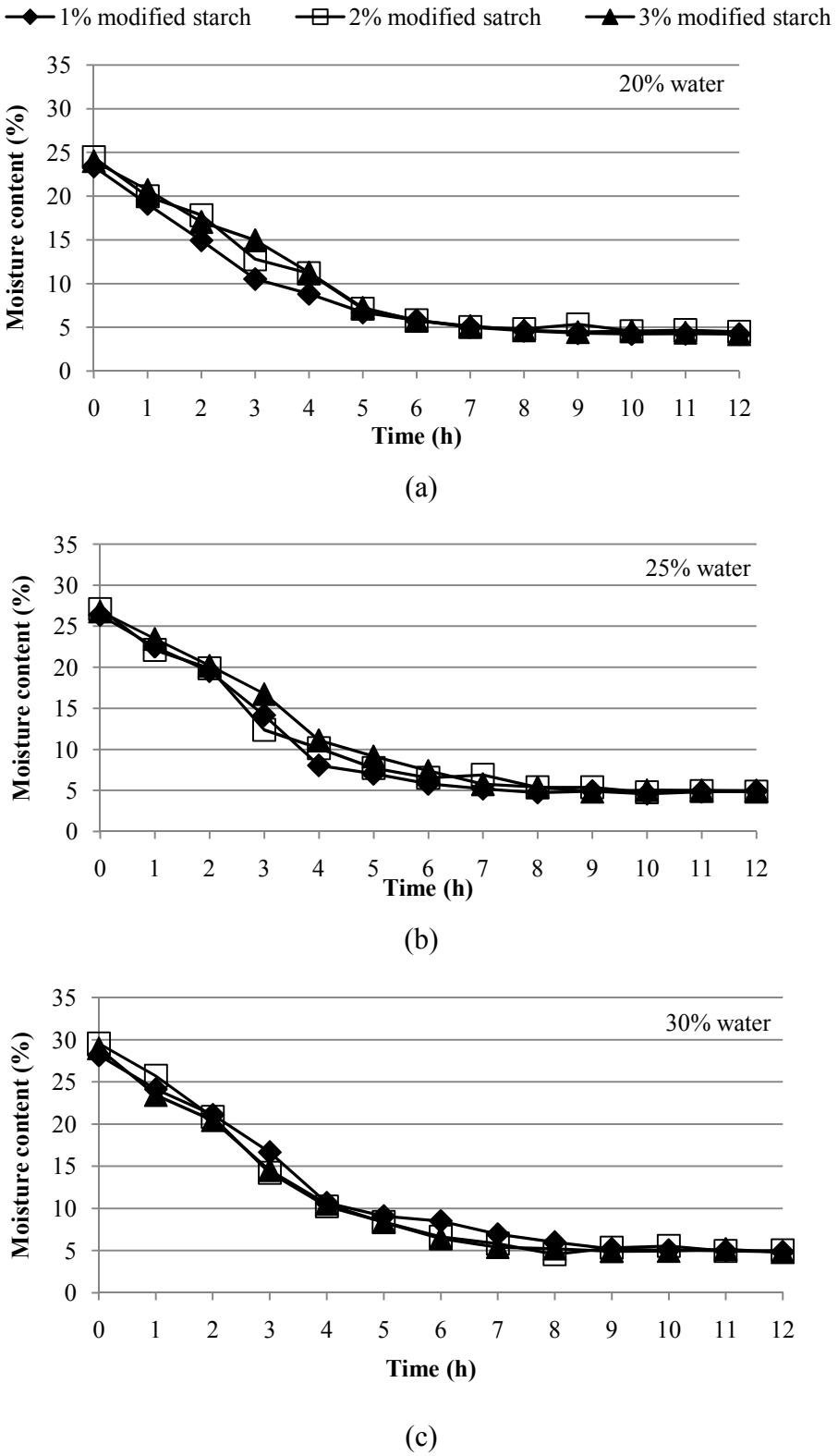


Figure 3 Drying curves of pelleted feed with various treatments undergoing drying at 50°C

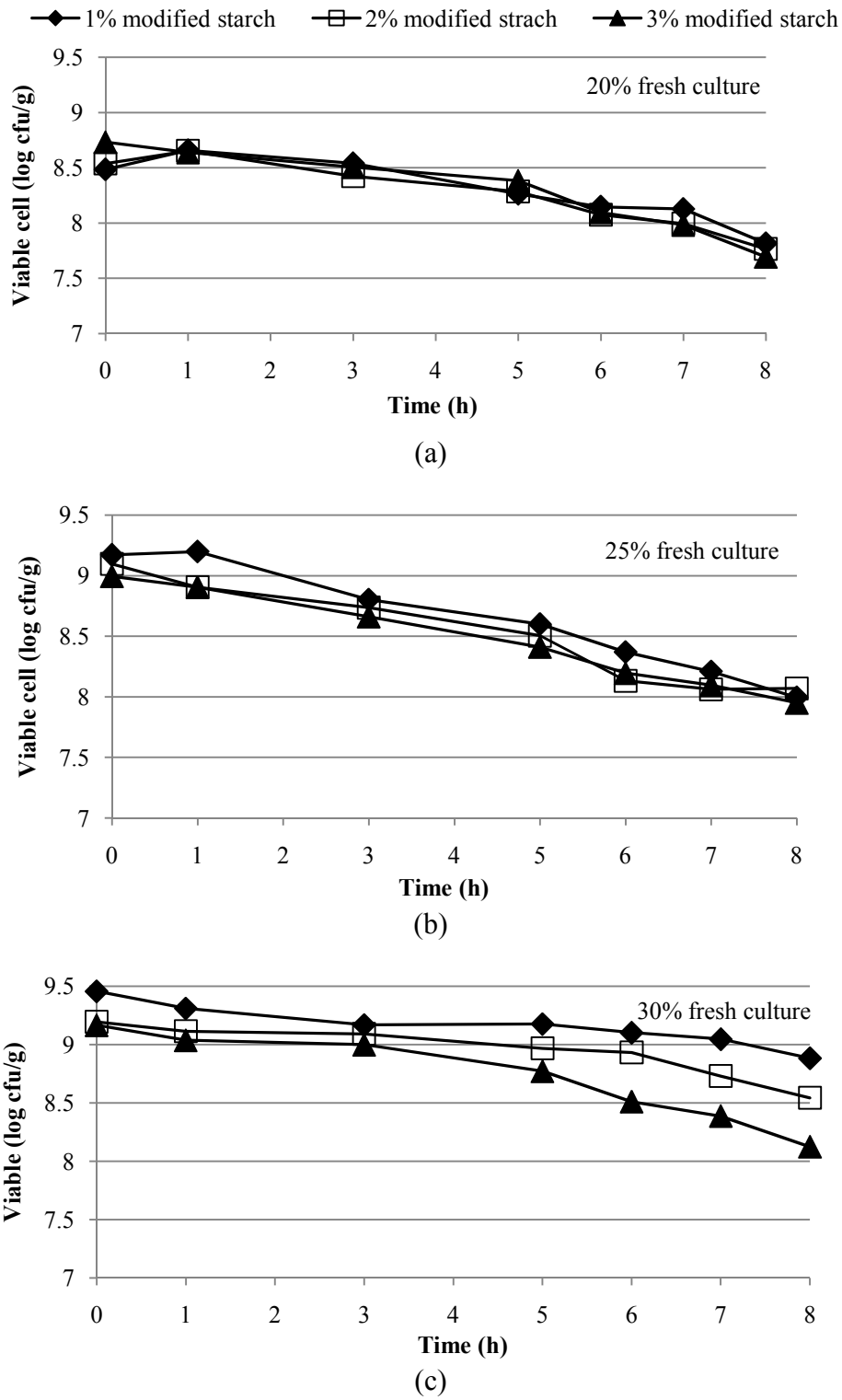


Figure 4 Viable cell number of *L. reuteri* KUB-AC5 in various pelleted feed treatments during drying at 40°C

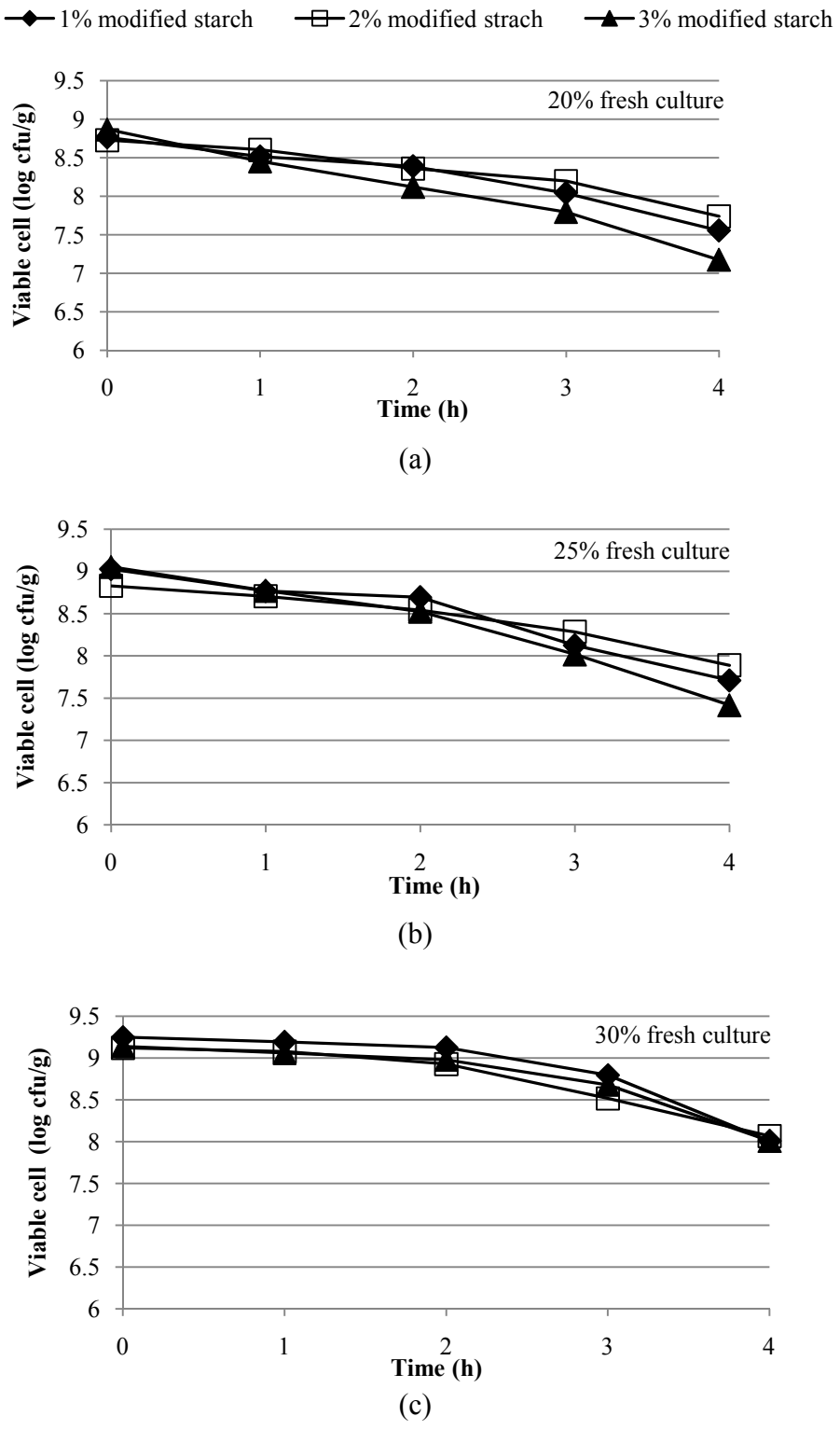


Figure 5 Viable cell number of *L. reuteri* KUB-AC5 in various pelleted feed treatments during drying at 50°C

The loss of viability *L. reuteri* KUB-AC5 during drying was quantitatively described in term of the specific rate of degradation (k) which was calculated by first order degradation kinetics as shown in Equation 1 (Desmons, 1998)

$$\log N = \log N_0 - kt \quad (1)$$

where N_0 is the number of initial viable cell (cfu/g of solids), N is the number of viable cell at any time of drying (cfu/g of solids), k is the specific rate of degradation (h^{-1}) and t is the drying time (h).

The results from Table 6 showed that the k was increased with increasing drying temperature. The degradation of viable cell during drying at 50°C was faster than drying at 40°C. The k was ranking from 0.0578 to 0.1511 h^{-1} (40°C) and 0.2290 to 0.4046 h^{-1} (50°C). These results were supported by Bayrock and Ingledew (1997). It was reported that the higher drying temperature decreased the viability of *S. cerevisiae* faster than lower drying temperature in the falling-rate drying period.

Table 6 Specific rate of degradation of *L. reuteri* KUB-AC5 in pelleted feed after drying at 40°C and 50°C

Fresh liquid culture	Specific rate of degradation (h^{-1})					
	40°C drying temperature			50°C drying temperature		
	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch
20%	0.0871	0.1000	0.1196	0.2892	0.238	0.4046
25%	0.1511	0.1372	0.1331	0.3276	0.2299	0.4033
30%	0.0578	0.0702	0.1218	0.2880	0.2664	0.2641

The increasing of viable cell due to temperature decreased was possible because of thermal inactivation (Johnson and Etzel, 1995) and it may be probably because probiotics are sensitive to temperature. Ananta *et al*. (2005) reported that the decreasing of viable cell after drying was most likely due to the removal of water which may cause irreversible changes in the structural and functional integrity of bacterial membranes and proteins. When cells were dried to low water content, a number of cell components such as DNA or RNA and proteins will be inflicted (Santivarangkna *et al*., 2008). However, the pelleted feed containing *L. reuteri* KUB-AC5 with a population of ca. 10^7-10^8 cfu/g were obtained after drying, meeting the number required for use as a probiotic product (Champagne *et al*., 2005).

Interestingly, the content of modified starch had significantly affected on the reduction of cell viability ($P<0.05$). The specific rate of degradation was increased with increasing modified starch content. The result revealed that optimum modified starch contents occurred in 1% and 2% of 40°C and 50°C pellet diet, respectively. Result from Figure 6 and 7, survival cell after drying was rather high, over than 6.6% and 2.0% for 40°C and 50°C, respectively. The highest survival of the strain (26.70%) was observed in 40°C dried pellet diet with 1% modified starch and 30% fresh liquid culture. For 50°C dried pellet treatments, pelleted feed with 2% modified starch and 25% fresh liquid culture gave the highest survival (11.28%).

2.1.2 Effect of modified starch and fresh culture on pellet hardness

The pellet hardness is an important quality which may play a role with preference of animals (Thomas and van der Poel, 1996). Hardness was determined by maximum loading (N). From Figure 8 and 9, the hardness of feed pellet was higher than commercial pelleted feed hardness (28.28 N). Hardness of pellet after drying at 40°C and 50°C were ranking from 56.30 N to 74.54 N and 54.16 N to 78.98 N, respectively. It was indicated that the hardness was significantly affected by modified starch contents ($P<0.05$). The pellet hardness was increased with increasing modified starch content. The pelleted feed with 3% modified starch had the highest hardness. Similar result was obtained by Thomas *et al*. (1998). Pregelatinized

starch as binder incorporated in the diets led to harder and more durable pellet. It was most likely because the surface tension of the binding liquid was higher than water (Thomas and van der Pole, 1996).

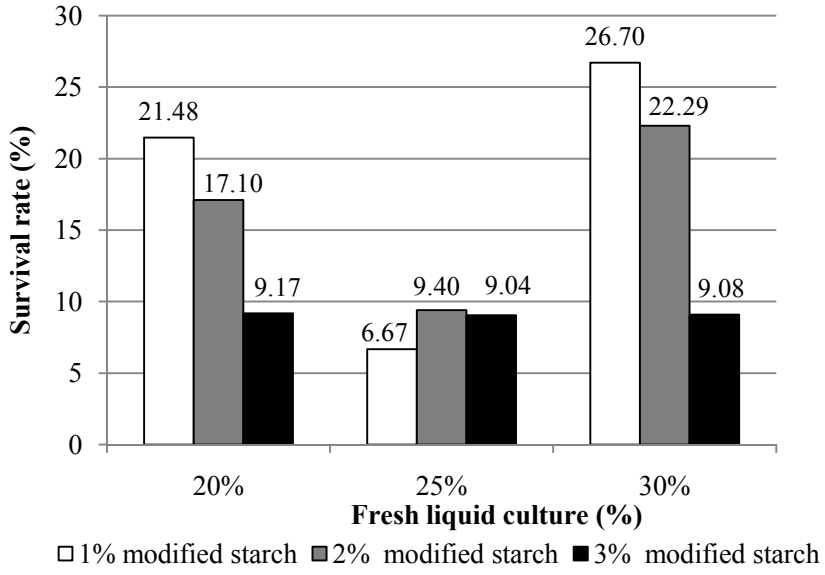


Figure 6 Survival of *L. reuteri* KUB-AC5 in pelleted feed after drying at 40°C

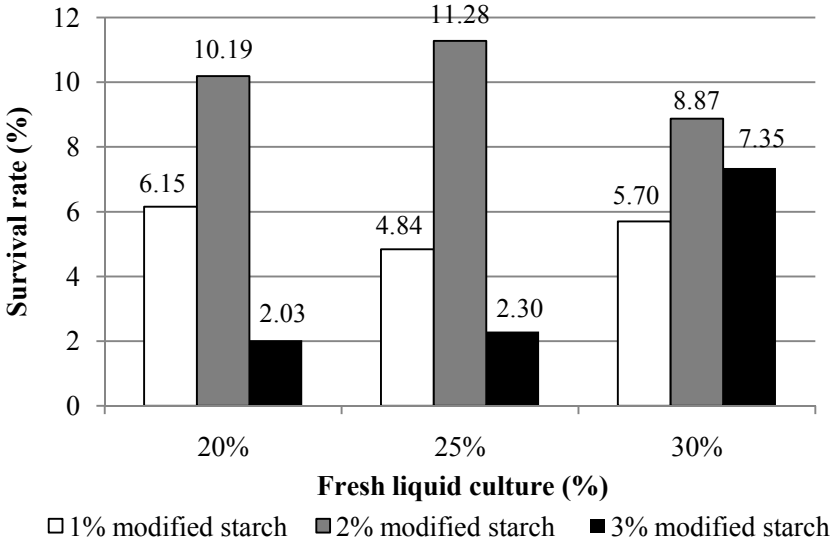


Figure 7 Survival of *L. reuteri* KUB-AC5 in pelleted feed after drying at 50°C

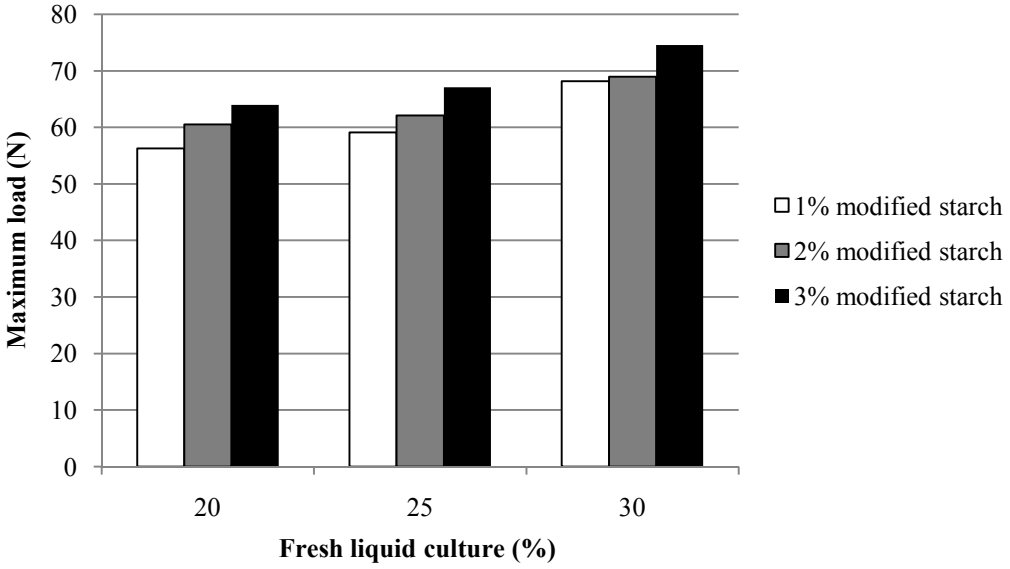


Figure 8 Pellet hardness of pelleted feed containing *L. reuteri* KUB-AC5 without protective agents after drying at 40°C

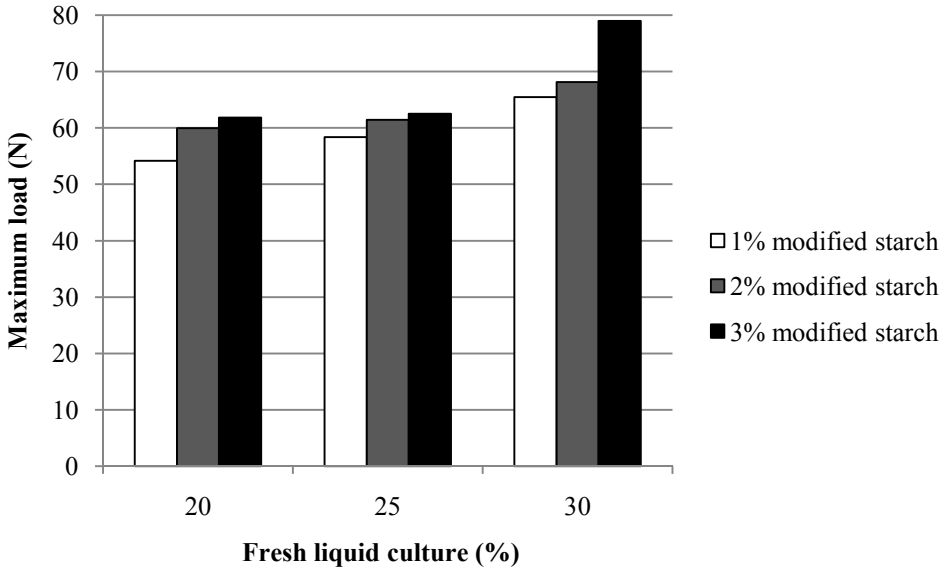


Figure 9 Pellet hardness of pelleted feed containing *L. reuteri* KUB-AC5 without protective agents after drying at 50°C

The modified starch has been used in the production of pellet as a filling and binding component for wet granulation process. The modified starch molecule was divided into two parts including soluble and insoluble. The soluble part of a molecule acts as a binding agent, while the insoluble part performs the role of filler possessing good loosening properties (Andreev, 2004). In pellets, binding of particles is a combination of solid-solid bonds between diet ingredient particles, capillary forces between water, air and solid material, adhesive and cohesive forces between ingredient particles and binders, and interactions between particles due to folding and plying. A combination binder with fresh water plays a role in capillary type binding as well as adhesive or cohesive forces (Thomas and van der Pole, 1996). Moreover, binders affect stability in three ways including; (1) to reduce void space in a more compact and durable pellet,(2) to act as adhesive sticking particles together and (3) to exert a chemical action on the ingredients and alter the nature of the feed resulting in a more durable pellet (Desilva and Anderson, 1995).

The contents of fresh liquid culture had also significantly affected on pellet hardness ($P<0.05$). The results revealed that pellet hardness increased with increasing the culture content. According to Moritz *et al*. (2002), the addition of 5% moisture into the diets was able to improve durability and decrease the percentage of fines. Water as the lower viscosity binder was generally added into the feed in order to provide sorption (Thomas *et al*., 1997). Water wet the hydrophilic substance and spread out from the core to form a looser network of extended pendular hydrate bonds, while water in the more viscous solution was less able to chemically interact with hydrophilic substance; the agglomerate retained a dense capillary core nucleus (Mort, 2005). In addition, it was found that pellet hardness did not depend on the drying temperature.

2.2 Production of pelleted feed containing *L. reuteri* KUB-AC5 with protective agents

Various protective agents have been investigated for protecting probiotic during dehydration for instance, skim milk powder, whey protein, monosodium

glutamate, sucrose, lactose and polymer such as dextran and polyethlylene glycol (Carvalho *et al*., 2003; Meng *et al*., 2008). In addition, various polymers have been used to encapsulate probiotic for enhancing the survival of cell during process and storage such as sodium alginate, carrageenan, chitosan, carboxymethyl cellulose (CMC) and starch (Muthukumarasamy *et al.*, 2006; Anal and Singh, 2007, Mortazavian *et al*., 2007). Thus, it may be useful to investigate the effect of protective agent on the viability of *L. reuteri* KUB-AC5 in pelleted feed during drying and subsequently storage.

2.2.1 Viability of *L. reuteri* KUB-AC5 with protective agent in pelleted feed during drying process

In this section, four different protective agents including milk powder (MP), monosodium glutamate (MSG), sodium alginate (SA) and carboxymethly cellulose (CMC) were investigated for their effects by comparing with the pelleted feed without protective agent (control).

Results from Figure 10 and 11 showed that the viability of *L. reuteri* KUB-AC5 was related to the type of protective agents. The loss of cell in both dried pellet treatment was approximately 1 log cycle excepted MSG at 50°C samples (ca. 2 log cycle). The reduction rate of the strain was quantitatively described by specific rate of degradation (k) as shown in Table 7. Milk powder gave the lowest specific rate of degradation of 0.0661 h^{-1} and 0.1709 h^{-1} during drying process at 40°C and 50°C respectively. In contrast, the sodium alginate of 40°C dried pellet displayed the lowest heat resistance (k = 0.1440 h^{-1}) while the poorest protective agent of 50°C dried pellet was obtained in pelleted feed with MSG (k = 0.4153 h^{-1}).

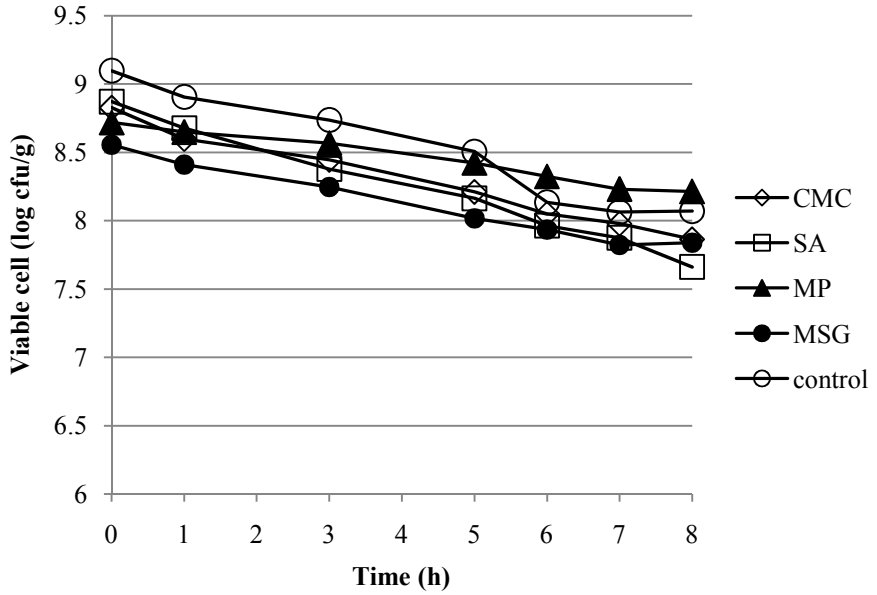


Figure 10 Viable cell of *L. reuteri* KUB-AC5 in pelleted feed with various protective agents during drying at 40°C

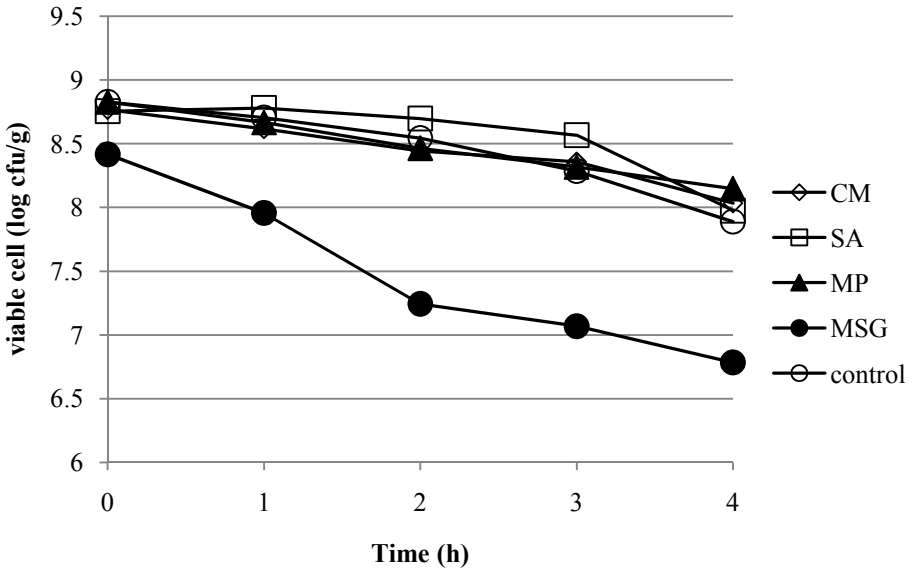


Figure 11 Viable cell of *L. reuteri* KUB-AC5 in pelleted feed with various protective agents during drying at 50°C

Table 7 Specific rate of degradation of *L. reuteri* KUB-AC5 in pelleted feed with various protective agents after drying at 40°C and 50°C

Protective agents	Specific rate of degradation, k (h^{-1})	
	40°C dried pelleted feed	50°C dried pelleted feed
Control	0.1181	0.2299
CMC	0.1178	0.1726
Sodium alginate	0.1440	0.1775
Milk powder	0.0661	0.1709
MSG	0.0944	0.4153

As shown in Figure 12, the survival of *L. reuteri* KUB-AC5 with CMC, sodium alginate, milk powder, MSG and control after drying at 40°C was 10.74%, 6.38%, 30.58%, 19.17% and 9.40%, respectively. *L. reuteri* KUB-AC5 with milk powder exhibited significantly highest survival degree ($P<0.5$). For 50°C drying, the survival of *L. reuteri* KUB-AC5 was ranking 2.33% to 20.73%. *L. reuteri* KUB-AC5 in milk powder treatment had the best survived (20.73%) while, the highest mortality was displayed MSG (2.33%). According to Hamsupo (2005), the survival of *L. reuteri* KUB-AC5 with MSG was the lowest survival cell after spray drying comparing with other carrier.

As the mention earlier, milk powder had potential to protect cell from heat. It was insisted by many researchers which the skim milk powder was as good protective agents during drying (Teixeira *et al*., 1995; Lian *et al*., 2002; Corcoran *et al*., 2004; Simpson *et al*, 2005). Moreover, Hamsupo (2005) found that viable cell number of *L. reuteri* KUB-AC5 with skim milk was slightly decreased after spray drying. The milk was able to protect cell probably because the protein of milk prevented cellular injury by stabilizing cell membrane constituents (Castro *et al*., 1995) and it may form a protective coating on the cell wall of the microorganism (King and Su, 1993). However, the various protective agents also had difference in chemical characteristics and physical properties such as thermal conductivity, thermal diffusivity, etc. Therefore, it is reason to expect that these protective agents tested in

the present study may exert different degree of protective effect on the survival of the strain.

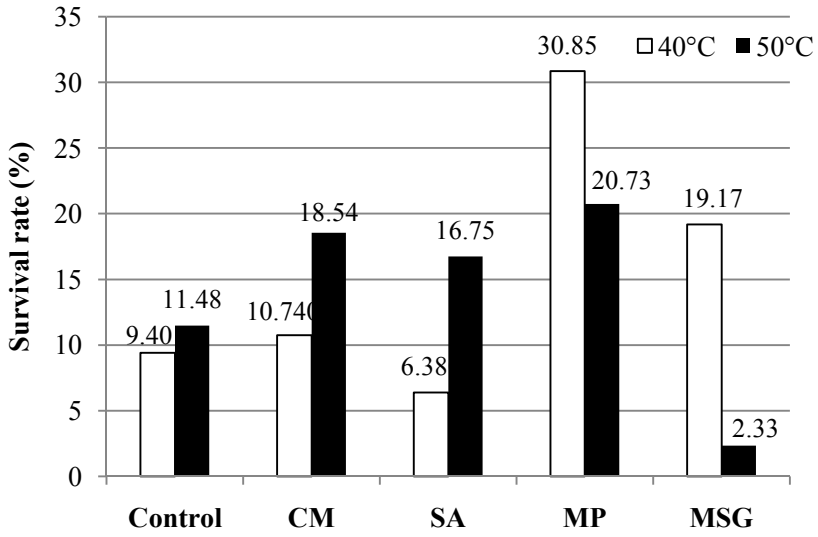


Figure 12 Survival of *L. reuteri* KUB-AC5 in pelleted feed with various protective agents after drying at 40°C and 50°C

2.2.2 Effect of protective agents on pellet hardness

From Figure 13, the protective agents selected had affected on hardness of pellet. The results showed that the hardness of all samples was higher than control. CMC gave the highest hardness which was 107.65 N and 107.81 N for 40°C and 50°C dried pellet, respectively and followed by sodium alginate (98.75 N for 40°C dried pellet and 95.55 for 50°C dried pellet). The increasing in pellet hardness may be possible because they had binding property. CMC binders as water-soluble polymers greatly affect the colloidal forces by adsorbing on feed particle. The dissolved polymers also make the liquid phase of a coating more viscous (Conceição *et al*., 2003). Sodium alginate acted as viscosifier and effective binder. It had gel-forming characteristic and structure building properties. The lowest pellet hardness was obtained by pelleted feed with MSG with pellets hardness of 75.44 N and 60.85 N

after drying at 40°C and 50°C, respectively. It was not statically difference with milk powder and control treatment (P<0.05).

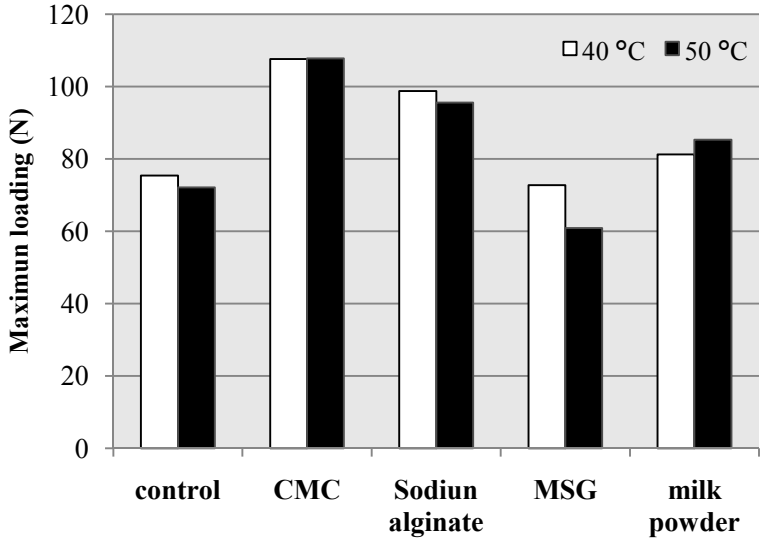
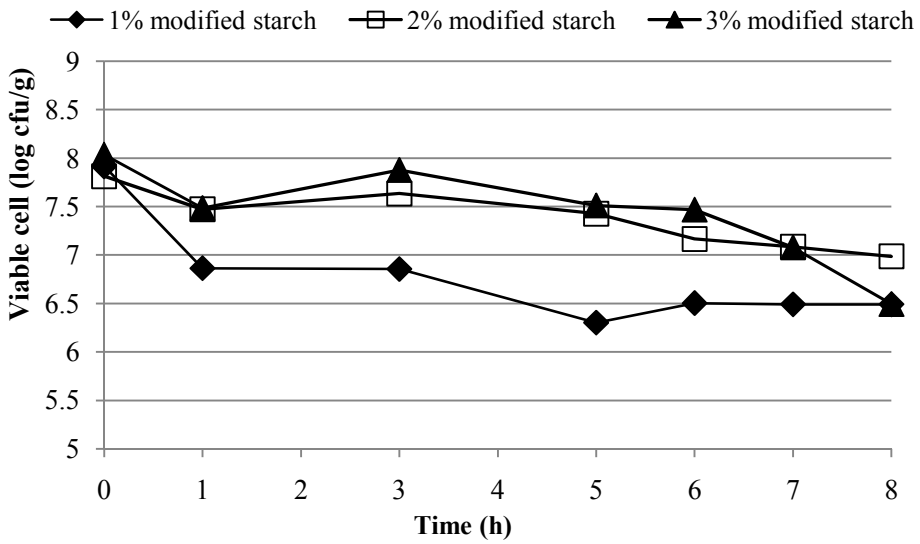


Figure 13 Hardness of diet pellet with various protective agents after drying at 40°C and 50°C

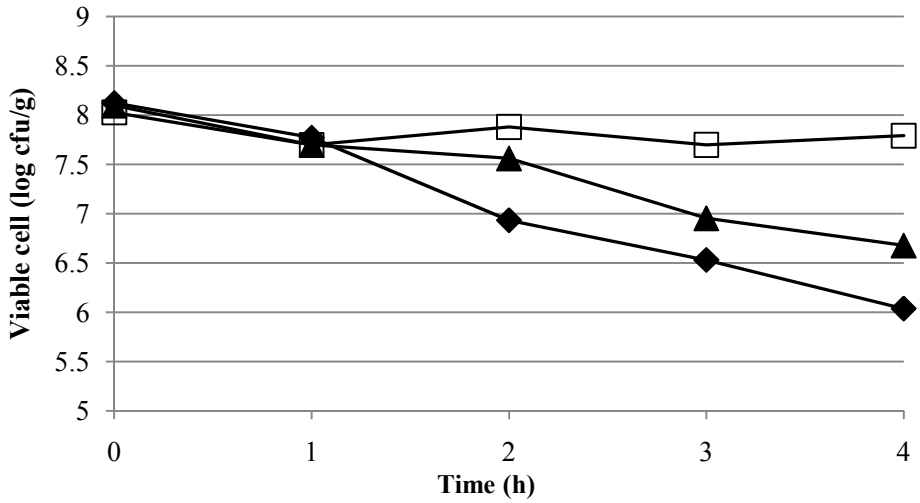
2.3 Production of pelleted feed containing spray-dried *L. reuteri* KUB-AC5

In this section, spray-dried *L. reuteri* KUB-AC5 (10^8cfu/g) was incorporated into broiler feed ingredients with various modified starch contents and water (25%v/w) prior to the pelleting process. Viability of the strain was investigated during drying process.

Figure 14 represented viable cell of spray-dried *L. reuteri* KUB-AC5 during drying at 40°C and 50°C. The results showed that the viable cell of all treatment was gradually decreased with increasing drying time. In all instances, the viable population of *L. reuteri* KUB-AC5 was decreased ca. 1–2 log cycles. After drying process, population of the strain in pelleted feed was of ca. 10^6-10^7 cfu/g.



(a)



(b)

Figure 14 Viable cell of spray-dried *L. reuteri* KUB-AC5 in pelleted feed during drying at 40°C (a) and 50°C (b)

The specific rate of degradation (k) of *L. reuteri* KUB-AC5 were investigated for effect of drying temperature and modified starch contents on viability of the strain during drying process (Table 8). In treatment of 1% and 3% modified starch, it was revealed that k was generally increased as increased drying temperature

while the 2% of modified starch exhibited the k lowest with 0.0915 h^{-1} and 0.0496 h^{-1} during drying at 40°C and 50°C, respectively.

Figure 15 illustrated, the survival of all treatments was over than 0.80%. Both 40°C and 50°C dried sample showed remarkably similar survival profiles in all treatments. The least mortality rate was observed in 2% modified starch, with survival of 15% and 58.21% after drying at 40°C and 50°C, respectively ($P<0.05$).

Table 8 Specific rate of degradation of *L. reuteri* KUB-AC5 in pelleted feed with spray-dried *L. reuteri* KUB-AC5 40°C and 50°C

Modified starch content	Specific rate of degradation, k (h^{-1})	
	40°C dried pelleted feed	50°C dried pelleted feed
1%	0.1429	0.5414
2%	0.0915	0.0469
3%	0.1381	0.3582

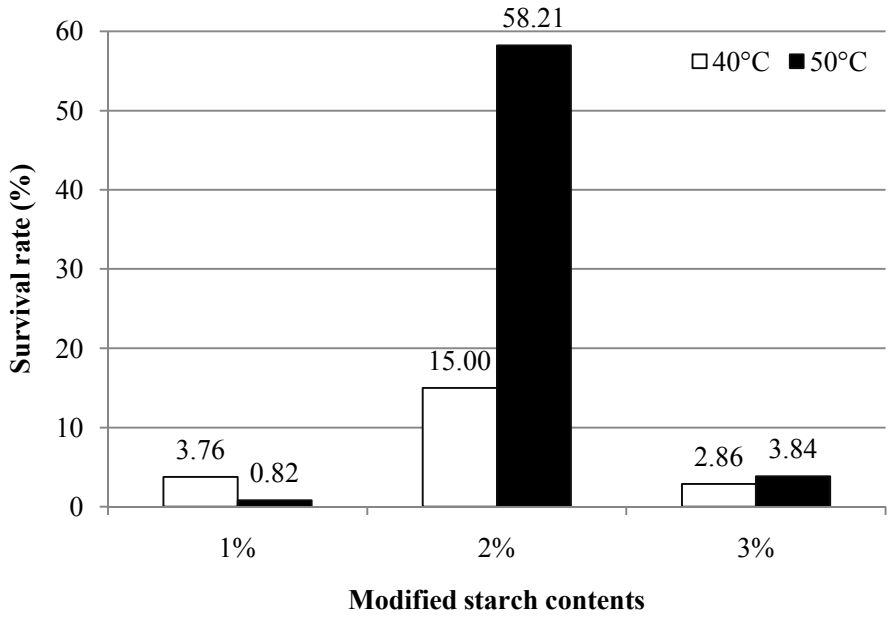


Figure 15 Survival of spray-dried *L. reuteri* KUB-AC5 in pelleted feed after drying at 40°C and 50°C

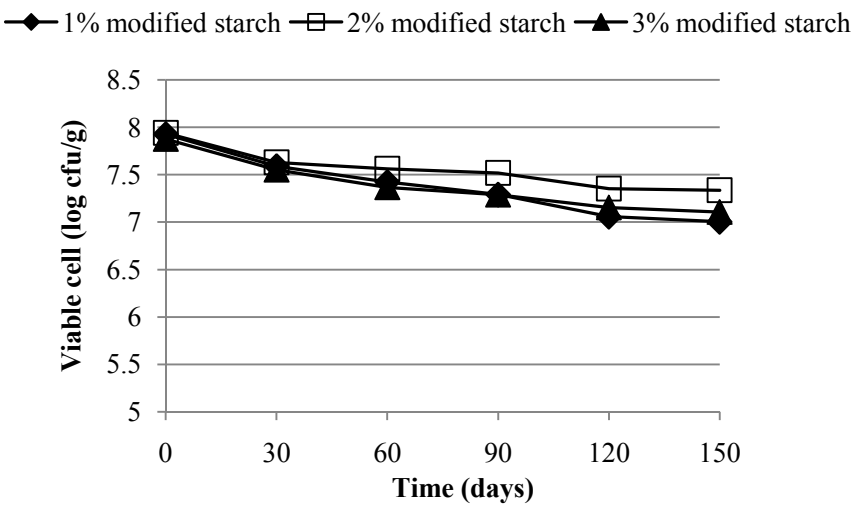
3. Stability of *L. reuteri* KUB-AC5 in pelleted feed during storage

The storage temperature has significant influence on the survival of probiotic and the correct storage conditions are essential to maintain viable populations of bacteria (Meng *et al*., 2008). The effects of storage temperatures on viability of *L. reuteri* KUB-AC5 in pelleted feed were evaluated in this section. Two temperatures (4°C and30°C) were tested for their effects on viability of the strain.

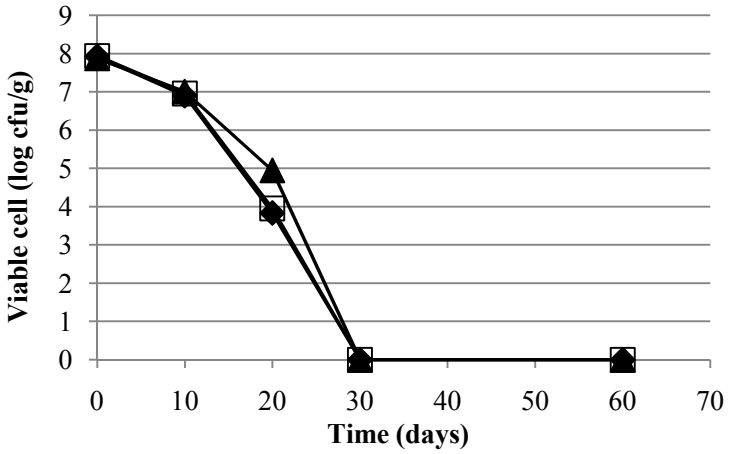
3.1 Survival of *L. reuteri* KUB-AC5 in pelleted feed during storage

Following drying process, the pelleted feed containing *L. reuteri* KUB-AC5 was placed in aluminum bag and stored at 4°C and 30°C for 150 days. For 40°C dried pellet diet, *L. reuteri* KUB-AC5 was quite stable and the viable cell number was gradually decreased ca. 1 log cycle (10^8 to 10^7 cfu/g) at 4°C storage (Figure 16a), while higher population reduction was observed when storage at 30°C. After 20 days of storage, viability of *L. reuteri* KUB-AC5 was still remained to 10^4-10^5cfu/g. However, the strain was completely lost after 30 days (Figure 16b).

For 50°C dried pellet diet, the result showed that *L. reuteri* KUB-AC5 in diet also survived well at 4°C (Figure 17a) and there was only 1.5 log cycles viable cell number reduction (10^7 to 10^5 cfu/g). While, high viability losses were observed at 30°C (Figure 17b). The mortality of *L. reuteri* KUB-AC5 was 10^3 to 10^4cfu/g after 20 days storage and absolutely lost after 30 days. The effect of modified starch on survival of *L. reuteri* KUB-AC5 during storage was subsequently assessed. Result showed that 2% modified starch exhibited significantly the lowest viable losses in both 40°C and 50°C ($P<0.05$).

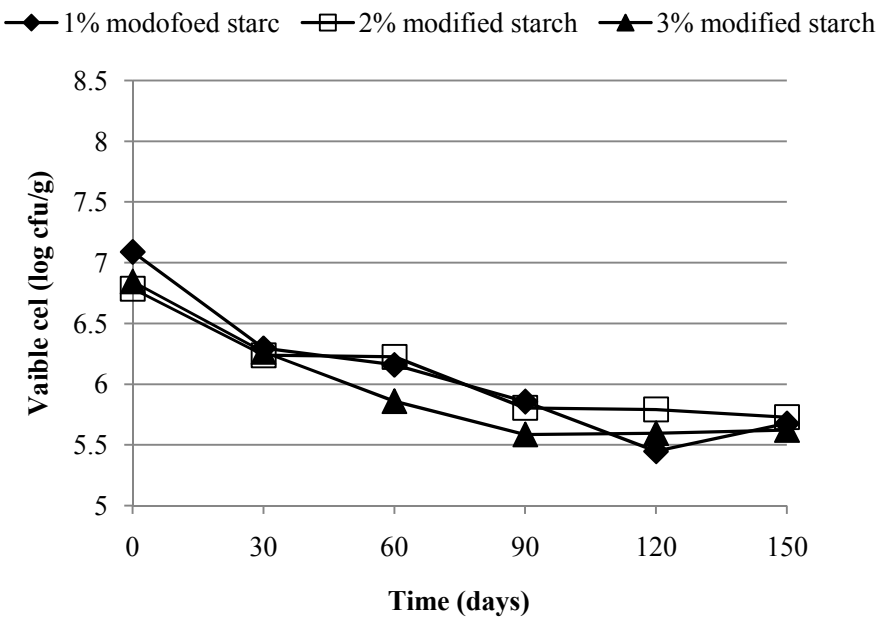


(a)

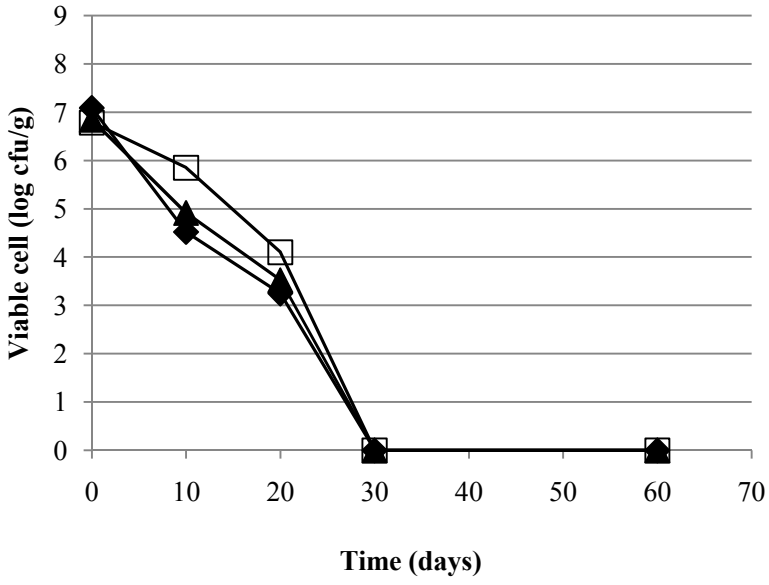


(b)

Figure 16 Viable cell of *L. reuteri* KUB-AC5 in 40°C dried pelleted feed during storage at 4°C (a) and 30°C (b)



(a)



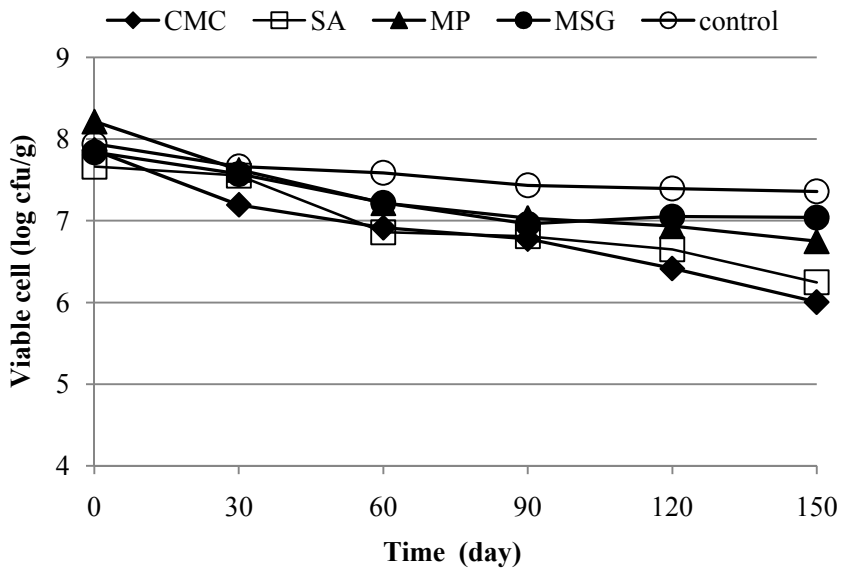
(b)

Figure 17 Viable cell of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed during storage at 4°C (a) and 30°C (b)

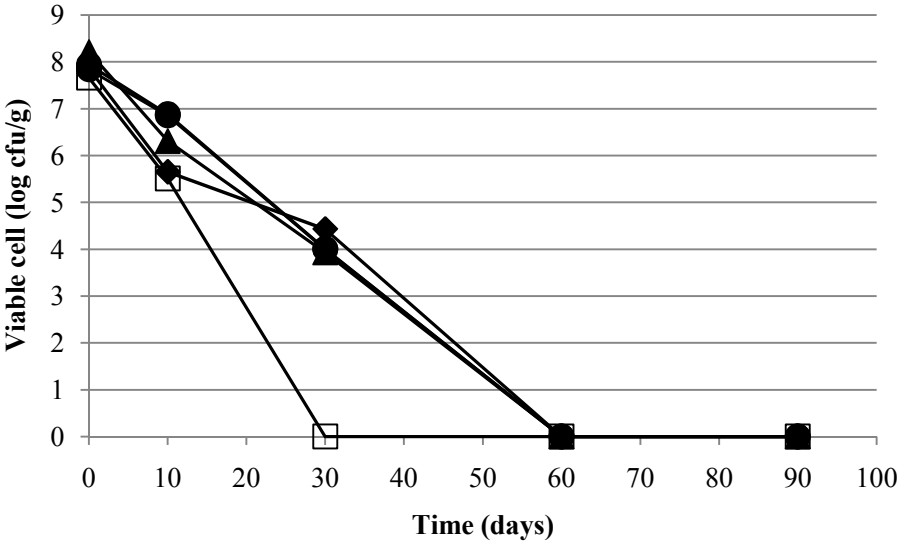
Effects of protective agents including CMC, sodium alginate (SA), milk powder (MP) and MSG on survival of *L. reuteri* KUB-AC5 were also investigated

during storage at 4°C and 30°C. As observed above, greatest the satin stability was obtained at storage lower temperature (Figure 18 and 19). For 40°C dried pellet diet after 150 days storage at 4°C, the result were difference from the expectance because *L. reuteri* KUB-AC5 experienced greatest viability stable in control (0.58 log cycle reduction), yielding the strain survival of 10^7cfu/g (Figure 18a) and followed by MSG (0.80 log cycle reduction), milk powder (1.18 log cycle reduction), sodium alginate (1.27 log cycle reduction) and CMC (1.38 log cycle reductions), respectively. However, the viable cell count in control was not significant difference with MSG ($P<0.05$). Similarly, the reduction of viable cell count in sodium alginate and milk powder also were not statically significant ($P<0.05$) During storage at 30°C, the least protection potential was observed in 40°C dried pellet diet with SA (Figure 18b). The viable cell was completely lost after 30 days storage. In contrast, the viable cell in other protective agents remained ca. of 10^4cfu/g, however the completely death of *L. reuteri* KUB-AC5 was obtained after 60 days storage.

L. reuteri KUB-AC5 also exhibited significantly highest viability in 50°C dried pellet diet with MSG during storage at 4°C for 150 days ($P<0.05$). The viable cell count was only decreased 0.75 log cycles (Figure 19a). While the survival of the strain occurred in milk powder was lower decreased 1.42 log cycles and it was not statically difference with control. Even more dramatic viability losses were obtained in SA and CMC treatment; there was 1.55 and 2.04 log cycle reduction in SA and CMC samples, respectively. These result displayed that CMC in 50°C dried pellet diet afforded the least protection. Furthermore, *L. reuteri* KUB-AC5 experienced the most cell death during storage at 30°C (Figure 19b). The mortality of the strain in 50°C dried pelleted feed was approximately 2 to 4 log cycles after storage for 30 days and *L. reuteri* KUB-AC5 was also absolutely lost as storage for 60 days.

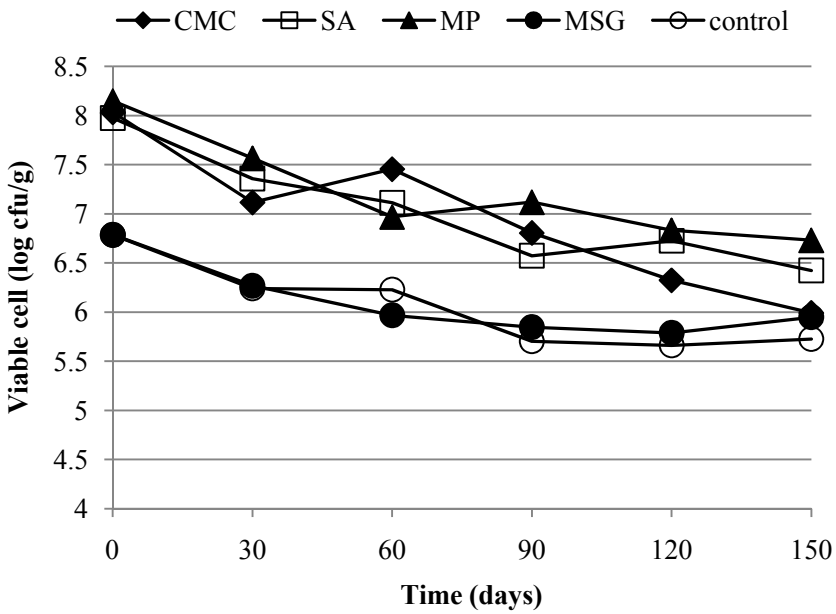


(a)

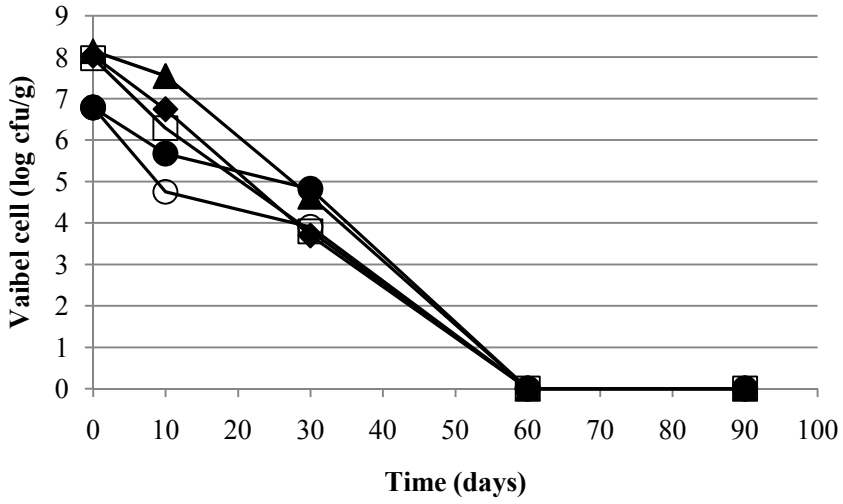


(b)

Figure 18 Viable cell of *L. reuteri* KUB-AC5 with various protective agents in 40°C dried pelleted feed during storage at 4°C (a) and 30°C (b)



(a)



(b)

Figure 19 Viable cell of *L. reuteri* KUB-AC5 with various protective agents in 50°C dried pelleted feed during storage at 4°C (a) and 30°C (b)

The impairment of viability during storage may be possibly related to oxygen diffusion into the dry cells though the interfacial area of the cell, because the cells remain permeable throughout storage. The accumulation of free radicals such as oxygen species within a cell that cannot metabolize them, or actively transport them

out of the cell, can result in irreversible damaging processes occurring within the cell (Morgan *et al*., 2006). For this reason, to reduce the effect of oxygen during storage, the presence of antioxidant in combination with protective agents was recommended. Teixeira *et al*. (1995) cited that the use of ascorbic acid as a well-know antioxidant together with monosodium glutamate increased the survival rates of *L. delbrueckii* ssp. *bulgaricus* during storage at 4°C. They believed that the ascorbic acid could have pro-oxidant properties as well and possibly produce hydroxyl radicals which can oxidize biological molecules.

The experimental results for the survival of *L. reuteri* KUB-AC5 in pelleted feed after storage at 4°C for 150 days was shown in Figure 20. The data showed that both 40°C and 50°C dried pellet diet gave the survival higher than 1.90%. The 2% of modified starch of both dried pellet afforded the highest survival, yielding *L. reuteri* KUB-AC5 survival of 30.32% and 8.36% for 40°C and 50°C dried pellet diet, respectively.

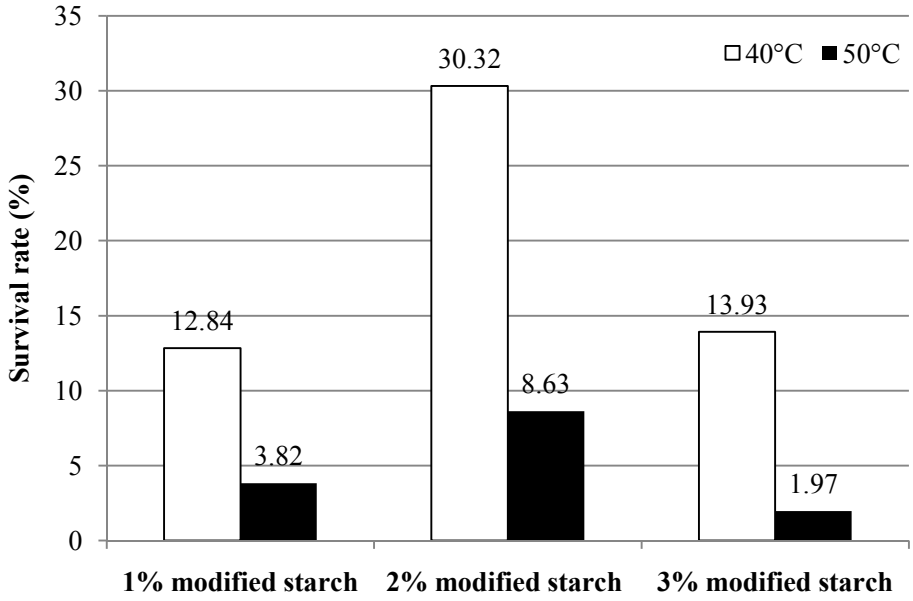


Figure 20 Survival of *L. reuteri* KUB-AC5 in dried pelleted feed after storage at 4°C for 150 days

Furthermore, effect of protective agents on survival of *L. reuteri* KUB-AC5 after storage at 4°C for 150 days was displayed as Figure 21. It was observed that the survival of all samples was ranking of 0.91% to 16.09%. The protective capacity of the evaluated protective agents in both 40°C and 50°C dried pellet diet increased in the following order: CMC < sodium alginate < milk powder < MSG, respectively.

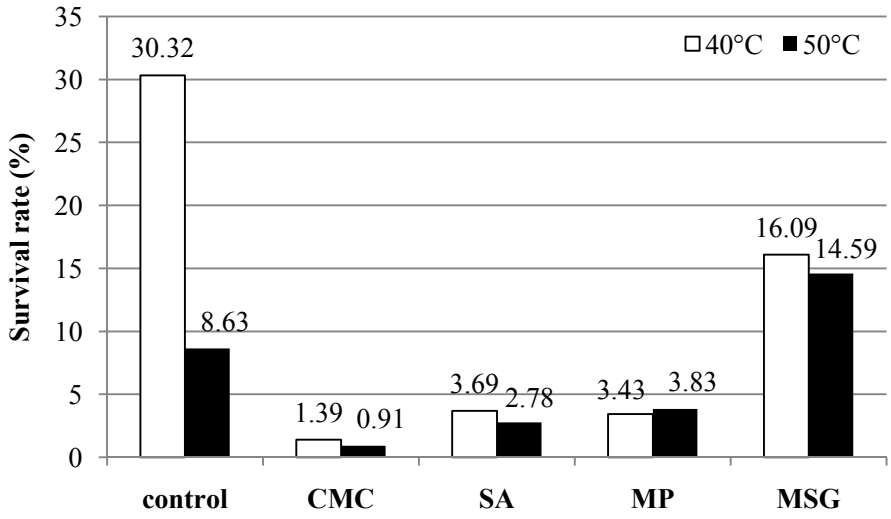


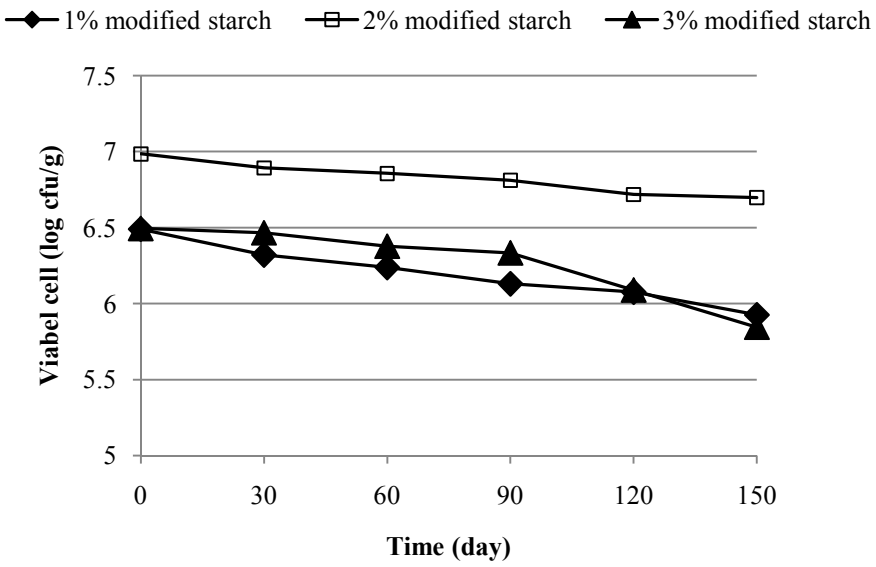
Figure 21 Survival of *L. reuteri* KUB-AC5 in dried pelleted feed with protective agents after storage at 4°C for 150 days

From mentioned earlier result, it was found that the storage temperature was a critical parameter. The viability of *L. reuteri* KUB-AC5 during storage was inversely related to storage temperature. The mortality of the strain was increased with decreasing temperature of storage. These obtained results were similar to many researchers who reported about probiotic viability during storage. Teixeira *et al.* (1995) studied survival of spray-dried *L. delbrueckii* ssp. *bulgaricus* during storage at difference temperature. They reported that the total of viable bacteria decreased as the storage temperature increased. Similarly, Selmer-Olsen *et al.* (1999) reported that survival of dried lactic acid bacteria was strongly dependent on the storage temperature. Viable cell number of probiotic was maintained ca. of 10^8cfu/g during storage at 4°C and 15°C for 49 days, whereas a storage temperature of 30°C was

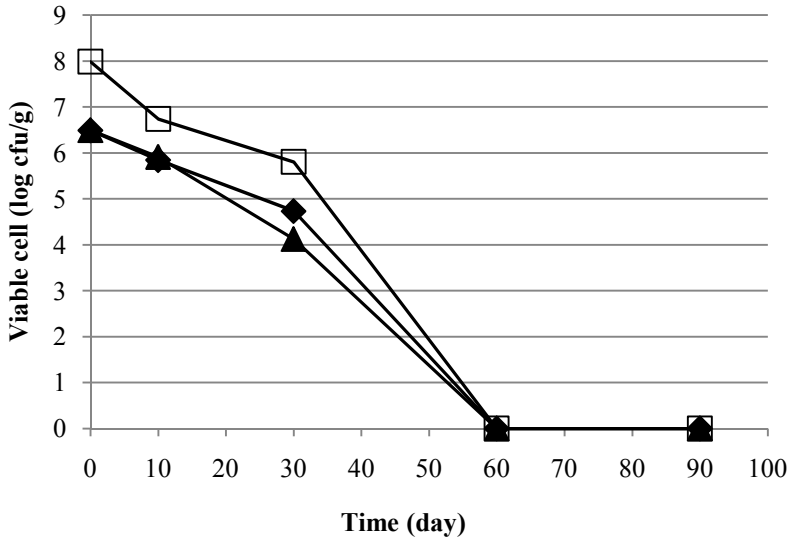
unsuitable, the viable cell declined to 10^6cfu/g during 49 days storage (Gardiner *et al*., 2002). Hamsupo (2005) cited that lethal thermal injury was the main factor involved in the reduction. Corcoran *et al*. (2004) demonstrated that a temperature maintained at 4°C was optimum for storage of spray-dried probiotics to maximize viability of *L. rhamnosus* GG. Furthermore, the viability of *L. plantarum* 44a in feed that stored in refrigeration and kept in vacuum packages was constant after 1 year storage. While feed was stored in glass flasks at higher temperature (25°C), viability of the strain started to decline by the second week. The viability of the strain was maintained in refrigeration due to bacteria metabolism decreases, and the accumulation of toxic wastes from the metabolism is likely minimized (Bucio *et al*., 2005). In addition, the result in examination of the effect of protective agent on viability of *L. reuteri* KUB-AC5 in pelleted feed was showed that MSG was the best protective agent. These similar results were reported by Carvalho *et al*. (2003), MSG was the efficient protectants which increased in residual activity and viability of lactic acid bacteria during drying. Moreover, MSG has a positive effect during storage of various dried lactic acid bacteria (Ferreira *et al*., 2005).

3.2 Survival of spray-dried *L. reuteri* KUB-AC5 in pelleted feed during storage

Under difference storage temperature (4°C and 30°C), the viable cell of spray-dried *L. reuteri* KUB-AC5 was represented as Figure 22 and 23. For 40°C dried pellet diet, the sample showed excellent stability during storage at 4°C for 150 days (Figure 22a). The viable cell of all samples was decreased lower than 1 log cycle. The ranking of viable cell reduction was between 0.29 and 0.70 log cycles. While, samples that were stored at 30°C showed a 1 log cycle reduction in bacteria count after 10 days of storage (Figure 23b). After 30 days storage, the population of *L. reuteri* KUB-AC5 was decreased ca. 2 log cycles and it was completely lost after storage for 60 days. Similarly, *L. reuteri* KUB-AC5 in 50°C dried pellet diet and stored at 4°C maintained high viability after 150 days of storage (Figure 23a). The mortality of *L. reuteri* KUB-AC5 in sample was ranking 0.56 to 1.04 log cycles.



(a)

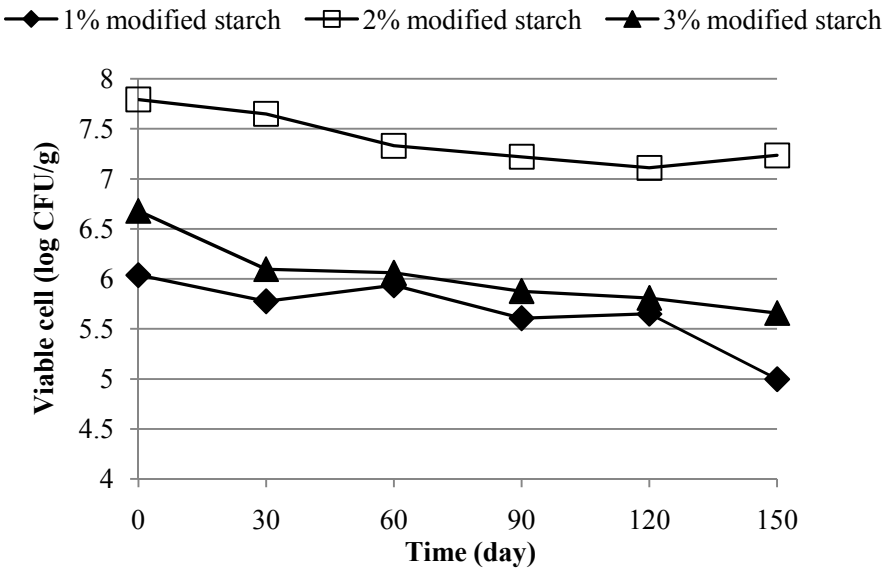


(b)

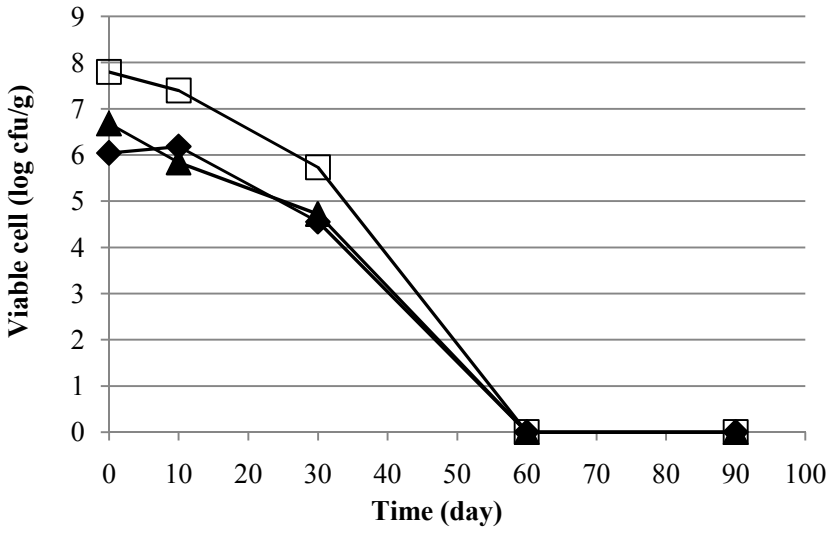
Figure 22 Viable cell of spray-dried *L. reuteri* KUB-AC5 in 40°C dried pelleted feed during storage at 4°C (a) and 30°C(b)

During storage at 30°C, the viable cell was dramatically decreased. The yielding probiotic survival was only 10^4-10^6 cfu/g after storage for 30 days and the viable cell absolutely lost within 60 days storage (Figure 23b). In addition, the effect of modified starch contents on cell survival was subsequently investigated. The

resulted revealed that 2% of modified starch in both 40°C and 50°C dried samples exhibited the greatest viable cell after storage at 4°C, with survival of 51.21% and 27.58% for 40°C and 50°C dried sample, respectively (Figure 24).



(a)



(b)

Figure 23 Viable cell of spray-dried *L. reuteri* KUB-AC5 in 50°C dried pelleted feed during storage at 4°C (a) and 30°C (b)

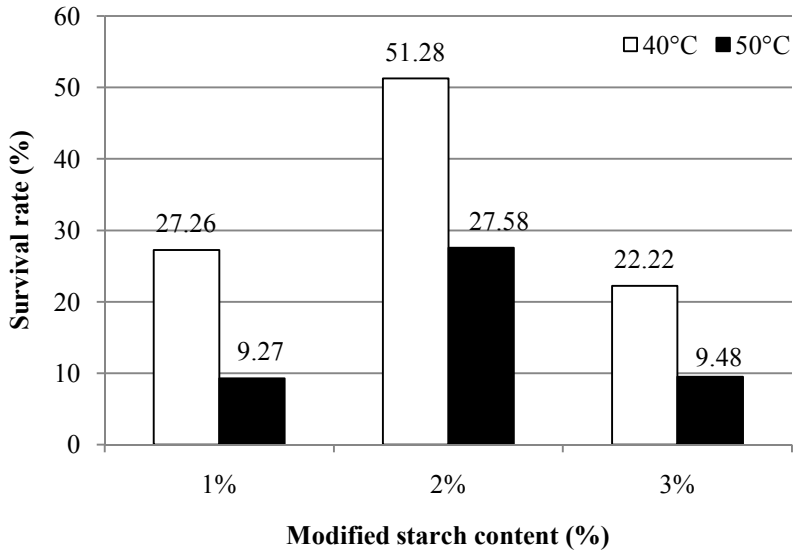
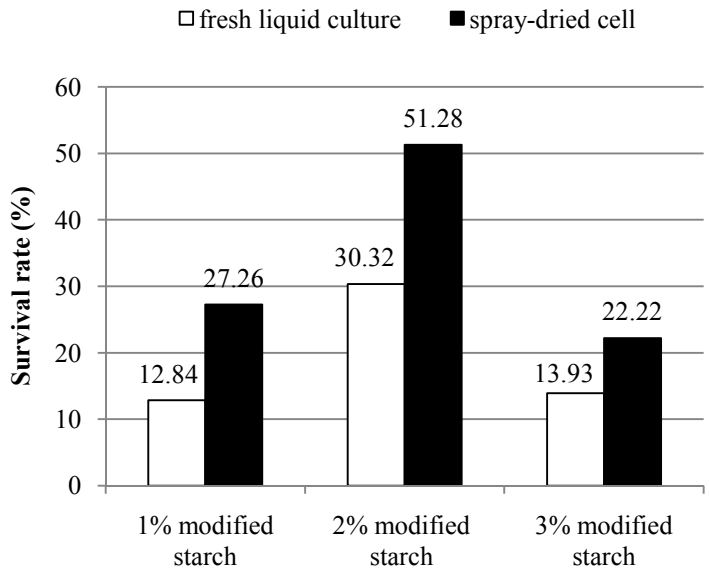
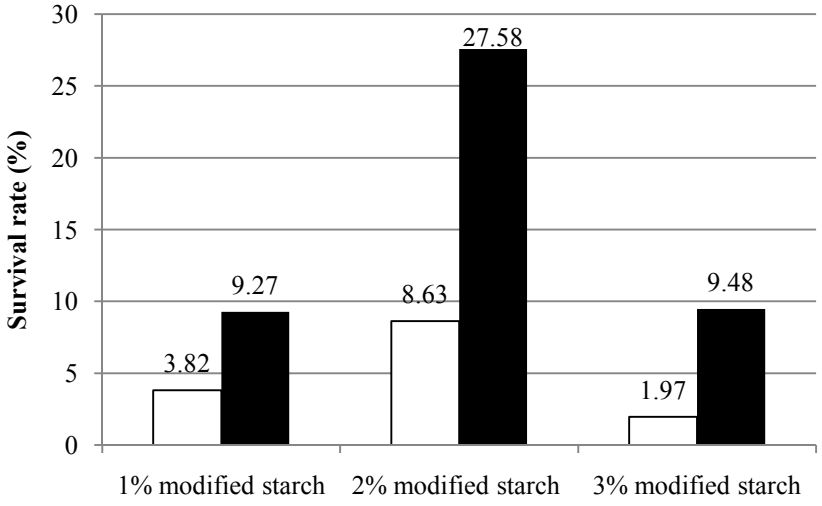


Figure 24 Survival of spray-dried *L. reuteri* KUB-AC5 in 40°C and 50°C dried pelleted feed after storage at 4°C for 150 days

Comparison on the survival of fresh liquid culture *L. reuteri* KUB-AC5 and spray-dried *L. reuteri* KUB-AC5 in the pelleted feed, it was indicated that addition of the strain in powder form gave survival better than cell suspension form during storage (Figure 25). It may be due to *L. reuteri* KUB-AC5 powder was coated by skim milk that creates a porous structure in the dried product and it is also believed that protein in the milk powder is a protective coating for the cells (Abadias *et al*, 2001). Desmond *et al.* (2002) reported that reconstituted skim milk (RSM) appears to be a very suitable media for efficacious spray-drying probiotic culture. Similarly, Cocoran *et al*. (2004) justified that skim milk was a suitable medium for large-scale production of shelf-stable spray-dried lactobacilli. Ananta *et al*. (2005) evaluated the effect of a spray-dried carrier on protection of *L. rhamnosus* GG at 25°C and 37°C storage condition and they found that RSM had the greatest protection capacity. The viable cell of spray-dried *L. rhamnosus* GG was decreased only 0.5 log cycle during storage at 37°C for 5 weeks. Hamsupo (2005) reported that the reduction of *L. reuteri* KUB-AC5 viable cell in skim milk powder was approximately 1 log cycle (from 10^9 to 10^8cfu/g) during storage at 4°C for 118 days.



(a)



(b)

Figure 25 Survival of fresh culture *L. reuteri* KUB-AC5 and spray-dried *L. reuteri* KUB-AC5 in 40°C (a) and 50°C (b) dried pelleted feed after storage at 4°C for 150 days

CONCLUSION

This study showed that it is possible to prepare probiotic pelleted feed with high number viable cell. Production of pelleted feed supplementing *Lactobacillus reuteri* KUB-AC5 was developed by using modified starch as binder and pelleting at room temperature. In practice, the pellet was dried at high temperature (up to 90°C) after pelleting process to reduce the moisture to lower 10% but it was the harsh condition for lactic acid bacteria. For this reason, drying temperature at either 40°C or 50°C was selected and the optimum drying time was investigated. After drying at 40°C and 50°C for 8 h and 4 h, respectively, the moisture content of ca. 8 % was obtained, which was close to the commercial feed moisture. Moreover, the pellet hardness was also measured. It was ranking from ca. 55 to 107 N depending on composition of feed which was 3-4 folds higher than of commercial feed (28.28 N).

L. reuteri KUB-AC5 could be prepared for use as fresh culture or spray-dried powder. The results of drying process demonstrated that the strain of either fresh culture or spray-dried *L. reuteri* KUB-AC5, directly incorporated into the feed prior to pelleting, was stable to the drying temperature. The remained viable cells of *L. reuteri* KUB-AC5 were ranking of 10^6-10^8cfu/g, which meet the number required for use as probiotic product. In addition, milk powder as protective agent was found to improve viability of the strain when the fresh culture was used.

Storage temperature was found to be a critical factor affecting the mortality of the cell. The results indicated that survival of *L. reuteri* KUB-AC5 in pelleted feed during storage was higher at lower temperature. During storage at 4°C, the survival of *L. reuteri* KUB-AC5 in all treatments was quite stable. The reduction of 1 log cfu/g was occurred after storage for 150 days. While, the viable cell number of the strain during storage at 30°C declined rapidly. It was completely lost within 30 days and addition of protective agents was found to extend the cell survival to 60 days.

Further research should be aimed at the industrial feasibility of the strain, to assess the capability of providing cost-effective and industrial-scale quantities for commercial

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APPENDICES

APPENDIX A

Pelleted Feed

Appendix Figure A1 Pellet mill

Appendix Figure A2 Pelleted feed containing *L. reuteri* KUB-AC5

APPENDIX B

Experimental result

Appendix Table B1 Moisture content of pelleted feed during drying at 40°C

Time (h)	Moisture content (%) of various treatment of pellet feed								
	20% water addition			25% water addition			30% water addition		
	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch
0	23.0447	23.5362	22.9081	25.9754	26.3025	25.5557	28.4631	29.0526	28.1522
1	21.1799	22.0174	22.3740	22.5340	24.3851	25.0715	26.0220	26.2203	26.3094
2	18.5837	20.3344	21.6295	18.4524	21.2354	21.9999	22.9508	24.6884	22.9541
3	15.8119	17.9304	19.5261	15.9156	18.6854	21.1964	17.6678	22.1322	21.5151
4	13.8839	17.3017	17.8839	13.6707	16.0354	20.7200	15.7629	20.1198	19.0446
5	12.0243	13.8740	14.9531	12.9354	15.3509	17.4068	11.7414	19.3258	14.6226
6	10.6841	11.2921	13.1415	10.9227	10.2824	13.9457	10.9949	16.0379	13.2104
7	8.9738	9.8966	11.4891	9.0918	9.1454	13.1629	9.3958	12.7615	11.9251
8	8.2566	8.9995	10.3249	8.3419	8.6771	10.5326	8.6935	9.5284	9.2765

Appendix Table B1 (Continued)

Time (h)	Moisture content (%) of various treatment of pellet feed								
	20% water addition			25% water addition			30% water addition		
	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch
9	7.9270	8.5057	8.8914	7.8306	7.9344	9.1662	8.1162	8.6992	8.1420
10	7.7053	8.2229	7.7247	7.5540	7.5844	9.3161	7.9445	8.9414	7.5958
11	7.2183	7.6585	7.4955	6.9308	7.0516	7.5802	7.4459	8.6335	6.9122
12	7.4596	7.4929	7.4995	7.4064	7.2060	7.4400	7.5749	7.7827	7.3420

Appendix Table B2 Moisture content of pelleted feed during drying at 50°C

Time (h)	Moisture content (%) of various treatment of pellet feed								
	20% water addition			25% water addition			30% water addition		
	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch
0	23.3992	24.4462	24.0015	26.3538	27.0647	26.8046	28.1621	29.5624	28.9870
1	19.0946	19.9762	20.7034	22.5112	22.0996	23.4675	24.1466	25.723	23.4317
2	14.9339	17.8335	17.0503	19.5247	19.875	20.1889	21.108	20.8481	20.4528
3	10.5204	12.7878	14.9512	14.158	12.3889	16.7462	16.6788	14.2338	14.5472
4	8.8172	11.1775	11.2654	8.0448	10.1482	11.1026	10.6657	10.2396	10.5481
5	6.6823	7.1249	7.2492	7.0333	7.7333	9.1553	9.0892	8.3976	8.3859
6	5.7925	5.8081	5.7613	5.8081	6.5294	7.3821	8.5134	6.6482	6.4515
7	5.0111	5.0311	5.1589	5.2002	6.8990	5.7756	6.9503	5.8442	5.4310
8	4.5976	4.7907	4.6651	4.7442	5.3844	5.3974	5.9982	4.5713	5.2481
9	4.3308	5.3396	4.4590	4.9279	5.3854	4.8627	5.2011	5.3566	4.9511
10	4.2260	4.5591	4.5822	4.5993	4.7784	5.0417	5.0570	5.5546	4.9573

Appendix Table B2 (Continued)

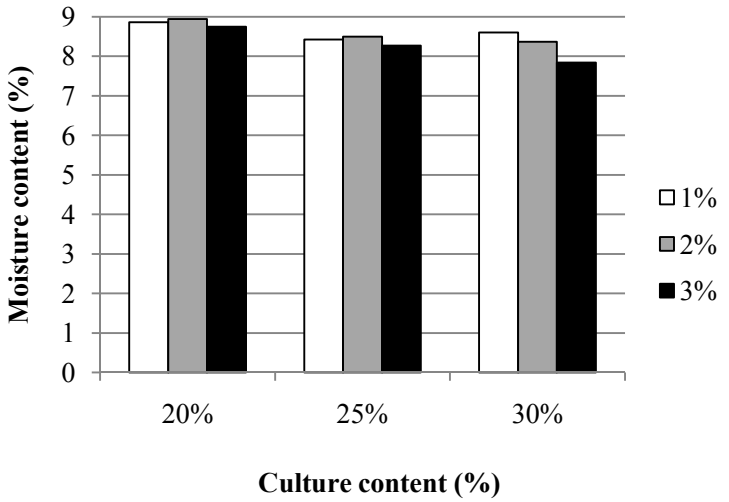
Time (h)	Moisture content (%) of various treatment of pellet feed								
	20% water addition			25% water addition			30% water addition		
	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch	1% modified starch	2% modified starch	3% modified starch
11	4.2491	4.6869	4.4060	4.8564	4.9214	5.0231	4.9983	4.9572	5.1947
12	4.2233	4.5024	4.2309	4.9493	4.8376	4.9772	4.8561	5.0272	4.8310

Appendix Table B3 Pellet hardness of pelleted feed containing of *L. reuteri* KUB-AC5 after drying at 40°C and 50°C

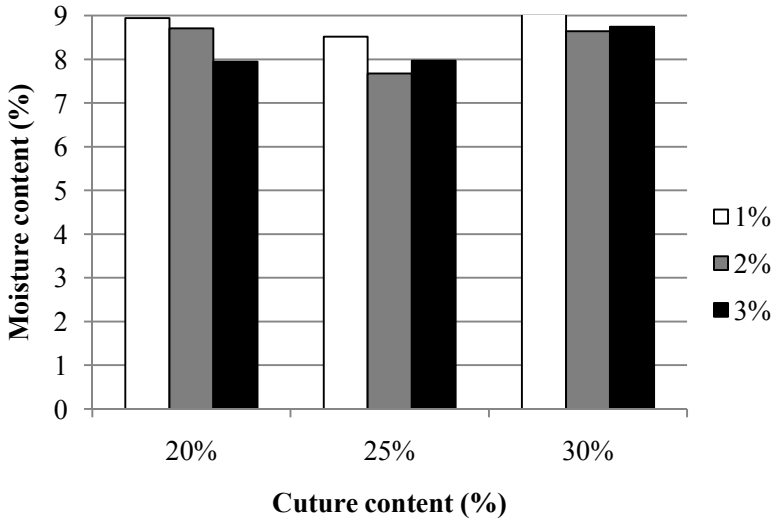
Modified starch content (%w/w)	Pellet hardness of pelleted feed with various culture content (N)					
	40°C dried pelleted feed			50°C dried pelleted feed		
	20%	25%	30%	20%	25%	30%
1%	56.30	59.08	68.18	54.16	58.36	65.48
2%	62.10	60.55	68.97	59.96	61.43	68.14
3%	63.95	67.11	74.54	61.81	62.49	78.98

Appendix Table B4 Moisture content of pelleted feed containing *L. reuteri* KUB-AC5 after drying at 40°C and 50°C

Modified starch content (%w/w)	Moisture content of pelleted feed with various culture content (%)					
	40°C dried pelleted feed			50°C dried pelleted feed		
	20%	25%	30%	20%	25%	30%
1%	8.862	8.423	8.607	8.940	8.520	9.483
2%	8.367	8.499	8.940	8.710	7.678	8.643
3%	7.842	8.270	8.746	7.946	7.965	8.741



Appendix Figure B1 Moisture content of pelleted feed containing of *L. reuteri* KUB-AC5 after drying at 40°C



Appendix Figure B2 Moisture content of pelleted feed with addition of *L. reuteri* KUB-AC5 after drying at 50°C

Appendix Table B5 Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed during drying at 40°C

Time (h)	Viable cell in pelleted feed with various modified starch contents (cfu/g)								
	20% culture			25% culture			30% culture		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
0	3.05E+08	3.45E+08	5.40E+08	1.49E+09	1.25E+09	9.90E+08	2.87E+09	1.57E+09	1.47E+09
1	4.55E+08	4.50E+08	4.40E+08	1.59E+09	8.05E+08	8.05E+08	2.05E+09	1.30E+09	1.10E+09
3	3.50E+08	2.67E+08	3.20E+08	6.35E+08	5.45E+08	4.60E+08	1.48E+09	1.24E+09	1.00E+09
5	1.84E+08	1.94E+08	2.41E+08	4.05E+08	3.20E+08	2.59E+08	1.51E+09	9.30E+08	6.05E+08
6	1.41E+08	1.19E+08	1.25E+08	2.35E+08	1.36E+08	1.58E+08	1.27E+09	8.55E+08	3.25E+08
7	1.34E+08	9.90E+07	9.60E+07	1.69E+08	1.16E+08	1.25E+08	1.12E+09	5.40E+08	2.45E+08
8	6.55E+07	5.90E+07	4.95E+07	9.90E+07	1.18E+08	8.95E+07	7.65E+08	3.50E+08	1.34E+08
Time (h)	Viable cell in pelleted feed with various modified starch contents (log cfu/g)								
	20% culture			25% culture			30% culture		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
0	8.484	8.537	8.731	9.171	9.097	8.995	9.457	9.195	9.167
1	8.658	8.653	8.638	9.199	8.906	8.905	9.311	9.114	9.037
3	8.540	8.424	8.505	8.803	8.736	8.663	9.169	9.092	9.000

Appendix Table B5 (Continued)

Time (h)	Viable cell in pelleted feed with various modified starch contents (log cfu/g)								
	20% culture			25% culture			30% culture		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
5	8.264	8.286	8.382	8.600	8.505	8.412	9.177	8.967	8.772
6	8.148	8.074	8.097	8.370	8.134	8.196	9.102	8.932	8.511

Appendix Table B6 Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed during drying at 50°C

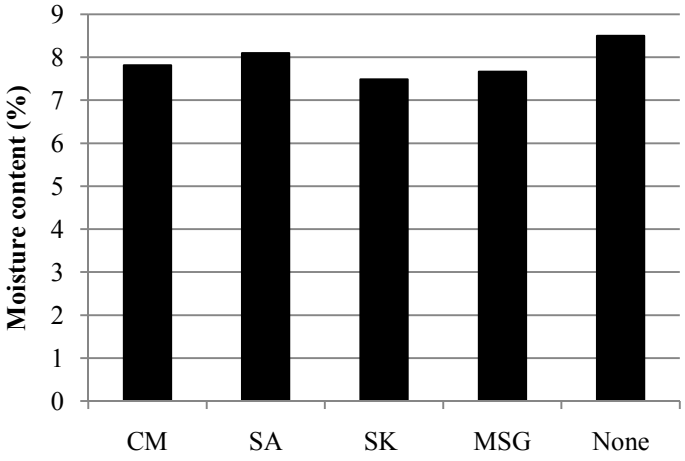
Time (h)	Viable cell in pelleted feed with various modified starch contents (CFU/g)								
	20% culture			25% culture			30% culture		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
0	5.85E+08	5.40E+08	7.40E+08	1.07E+09	6.75E+08	1.14E+09	1.79E+09	1.33E+09	1.40E+09
1	3.30E+08	4.10E+08	2.85E+08	5.85E+08	5.10E+08	5.95E+08	1.57E+09	1.20E+09	1.17E+09
2	2.45E+08	2.29E+08	1.38E+08	4.95E+08	3.50E+08	3.35E+08	1.34E+09	8.45E+08	9.60E+08
3	1.11E+08	1.58E+08	6.25E+07	1.34E+08	1.93E+08	1.04E+08	6.25E+08	3.30E+08	4.80E+08
4	3.60E+07	5.50E+07	1.50E+07	5.15E+07	7.75E+07	2.61E+07	1.02E+08	1.18E+08	1.03E+08
Time (h)	Viable cell in pelleted feed with various modified starch contents (log cfu/g)								
	20% culture			25% culture			30% culture		
	1%	2%	3%	1%	2%	3%	1%	2%	3%
0	8.764	8.728	8.869	9.027	8.828	9.054	9.249	9.121	9.141
1	8.518	8.602	8.454	8.767	8.706	8.773	9.194	9.077	9.060
2	8.389	8.359	8.122	8.694	8.544	8.525	9.126	8.927	8.980
3	8.042	8.197	7.796	8.125	8.285	8.017	8.795	8.519	8.681
4	7.556	7.740	7.175	7.710	7.889	7.416	8.009	8.068	8.010

Appendix Table B7 Pellet hardness of pelleted feed containing of *L. reuteri* KUB-AC5 with various protective agents after drying at 40°C and 50°C

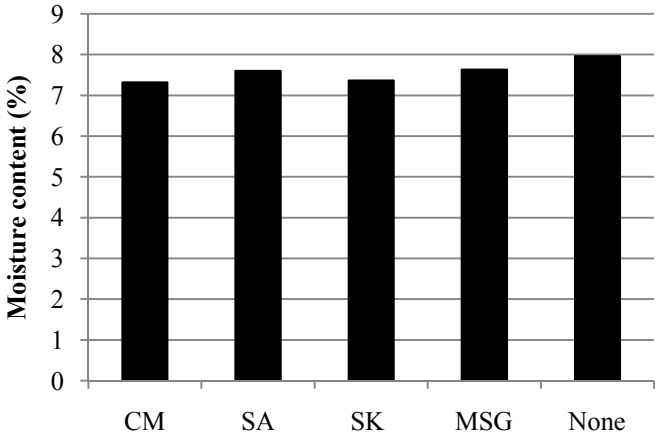
Protective agents	Pellet hardness of pelleted feed with various culture content (N)	
	40°C dried pelleted feed	50°C dried pelleted feed
None	75.44	72.08
CMC	107.65	107.81
Sodium alginate	98.75	95.55
Milk powder	81.26	85.33
MSG	72.71	60.85

Appendix Table B8 Moisture content of pelleted feed with various protective agents during drying at 40°C and 50°C

Protective agent	Moisture content (%)	
	40°C dried pelleted feed	50°C dried pelleted feed
None	8.499	7.965
CMC	7.819	7.318
Sodium alginate	8.097	7.603
Milk power	7.490	7.365
MSG	7.670	7.633



Appendix Figure B3 Moisture content of pelleted feed with addition of *L. reuteri* KUB-AC5 and various protective agents after drying at 40°C for 8 h



Appendix Figure B4 Moisture content of pelleted feed with addition of *L. reuteri* KUB-AC5 and various protective agents after drying at 50°C for 4 h

Appendix Table B9 Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent during drying at 40°C

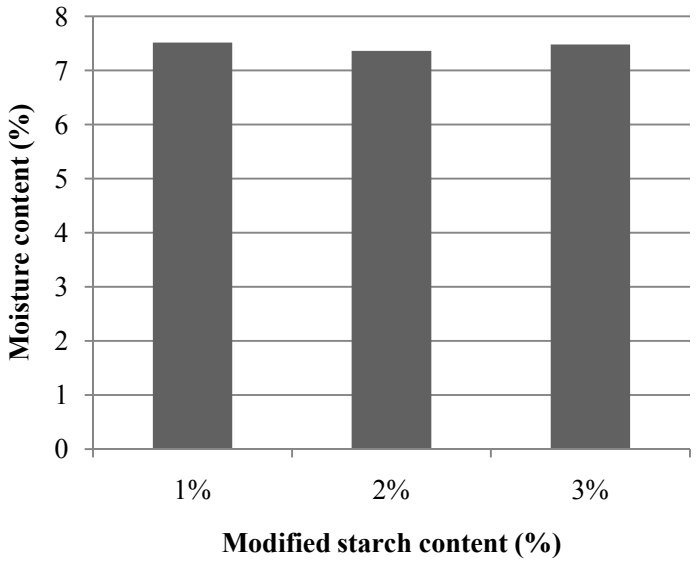
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent (cfu/g)				
	CMC	sodium alginate	milk powder	MSG	none
0	6.80E+08	7.45E+08	5.30E+08	3.60E+08	1.25E+09
1	4.05E+08	4.75E+08	4.65E+08	2.58E+08	8.05E+08
3	2.79E+08	2.39E+08	3.70E+08	1.77E+08	5.45E+08
5	1.63E+08	1.46E+08	2.66E+08	1.04E+08	3.20E+08
6	1.13E+08	9.20E+07	2.10E+08	8.60E+07	1.36E+08
7	9.50E+07	7.50E+07	1.70E+08	6.65E+07	1.16E+08
8	7.30E+07	4.75E+07	1.64E+08	6.90E+07	1.18E+08
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent (log cfu/g)				
	CMC	sodium alginate	milk powder	MSG	none
0	8.827	8.871	8.718	8.556	9.097
1	8.598	8.676	8.648	8.411	8.906
3	8.446	8.378	8.567	8.246	8.736
5	8.211	8.164	8.422	8.017	8.505
6	8.049	7.964	8.322	7.934	8.134
7	7.978	7.875	8.230	7.823	8.062
8	7.863	7.661	8.213	7.838	8.070

Appendix Table B10 Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent during drying at 50°C

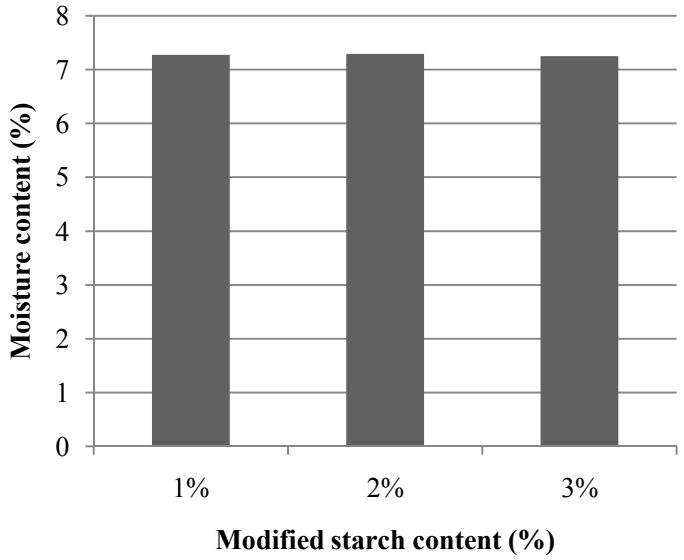
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent (cfu/g)				
	CMC	sodium alginate	milk powder	MSG	none
0	5.85E+08	5.70E+08	6.80E+08	2.62E+08	6.75E+08
1	4.15E+08	6.20E+08	4.65E+08	9.05E+07	5.10E+08
2	2.78E+08	5.00E+08	2.92E+08	1.76E+07	3.50E+08
3	2.28E+08	3.70E+08	2.08E+08	1.17E+07	1.93E+08
4	1.09E+08	9.55E+07	1.41E+08	6.10E+06	7.75E+07
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with addition of various protective agent (log cfu/g)				
	CMC	sodium alginate	milk powder	MSG	none
0	8.77	8.76	8.83	8.417	8.828
1	8.62	8.78	8.67	7.956	8.706
2	8.44	8.70	8.46	7.243	8.544
3	8.36	8.57	8.32	7.069	8.285
4	8.03	7.97	8.15	6.784	7.889

Appendix Table B11 Moisture content of pelleted feed with spray dried *L. reuteri* KUB-AC5 after drying at 40°C and 50°C

Modified starch content (%w/w)	Moisture content (%)	
	40°C dried pelleted feed	50°C dried pelleted feed
1%	7.512	7.274
2%	7.363	7.295
3%	7.479	7.249



Appendix Figure B5 Moisture content of pelleted feed with addition spray dried *L. reuteri* KUB-AC5 after drying at 40°C



Appendix Figure B6 Moisture content of pelleted feed with addition spray dried *L. reuteri* KUB-AC5 after drying at 50°C

Appendix Table B12 Viable cell number of spray-dried *L. reuteri* KUB-AC5 in pelleted feed during drying at 40°C

Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with various modified starch content (cfu/g)		
	1% modified starch	2% modified starch	3% modified starch
0	8.25E+07	6.50E+07	1.10E+08
1	7.30E+06	2.97E+07	3.05E+07
3	7.20E+06	4.30E+07	7.60E+07
5	2.00E+06	2.67E+07	3.25E+07
6	3.10E+06	1.22E+07	1.20E+07
7	3.10E+06	9.75E+06	3.15E+06
8	8.25E+07	6.50E+07	1.10E+08
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with various modified starch content (log cfu/g)		
	1% modified starch	2% modified starch	3% modified starch
0	7.92	7.81	8.04
1	6.86	7.47	7.48
3	6.85	7.63	7.88
5	6.30	7.43	7.51
6	6.50	7.17	7.47
7	6.49	7.09	7.08
8	6.49	6.99	6.50

Appendix Table B13 Viable cell number of spray-dried *L. reuteri* KUB-AC5 in pelleted feed during drying at 50°C

Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with various modified starch content (cfu/g)		
	1% modified starch	2% modified starch	3% modified starch
0	1.33E+08	1.07E+08	1.25E+08
1	1.33E+07	5.95E+07	5.00E+07
2	8.60E+06	7.60E+07	3.65E+07
3	3.40E+06	5.00E+07	9.00E+06
4	1.10E+06	6.20E+07	4.80E+06
Time (h)	Viable cell number of *L. reuteri* KUB-AC5 in pelleted feed with various modified starch content (log cfu/g)		
	1% modified starch	2% modified starch	3% modified starch
0	8.12	8.03	8.10
1	7.77	7.70	7.70
2	6.93	7.88	7.56
3	6.53	7.70	6.95
4	6.04	7.79	6.68

Appendix Table B14 Viable cell number of *L. reuteri* KUB-AC5 in 40°C dried pelleted feed with various treatments during at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
1% modified starch	8.45E+07	3.95E+07	2.64E+07	1.95E+07	1.14E+07	1.01E+07
2% modified starch	8.70E+07	4.25E+07	3.65E+07	3.30E+07	2.25E+07	2.16E+07
3% modified starch	7.50E+07	3.55E+07	2.33E+07	1.94E+07	1.43E+07	1.28E+07
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
1% modified starch	7.926	7.589	7.420	7.289	7.055	7.002
2% modified starch	7.939	7.628	7.562	7.518	7.352	7.334
3% modified starch	7.875	7.550	7.364	7.286	7.153	7.105

Appendix Table B15 Viable cell number of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed during storage at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
1% modified starch	1.23E+07	1.98E+06	1.45E+06	7.20E+05	2.85E+05	4.75E+05
2% modified starch	6.20E+06	1.73E+06	1.68E+06	6.40E+05	6.20E+05	5.35E+05
3% modified starch	7.10E+06	1.85E+06	7.25E+05	3.85E+05	3.95E+05	4.20E+05
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
1% modified starch	7.090	6.296	6.159	5.857	5.447	5.676
2% modified starch	6.787	6.238	6.225	5.805	5.792	5.727
3% modified starch	6.846	6.265	5.860	5.585	5.595	5.622

Appendix Table B16 Viable cell number of *L. reuteri* KUB-AC5 in 40°C dried pelleted feed during storage 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (CFU/g)					
	0	10	20	30	60	90
1% modified starch	8.45E+07	8.00E+06	6.95E+04	0	0	0
2% modified starch	8.70E+07	9.15E+06	4.74E+04	0	0	0
3% modified starch	7.50E+07	1.01E+07	8.85E+03	0	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (CFU/g)					
	0	10	20	30	60	90
1% modified starch	7.926	6.903	3.835	0	0	0
2% modified starch	7.939	6.960	3.958	0	0	0
3% modified starch	7.875	7.003	4.946	0	0	0

Appendix Table B17 Viable cell number of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed during storage at 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (CFU/g)					
	0	10	20	30	60	90
1% modified starch	1.23E+07	3.35E+04	1.78E+03	0	0	0
2% modified starch	6.20E+06	7.20E+05	1.26E+04	0	0	0
3% modified starch	7.10E+06	8.30E+04	3.35E+03	0	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (CFU/g)					
	0	10	20	30	60	90
1% modified starch	7.090	4.521	3.249	0	0	0
2% modified starch	6.787	5.850	4.100	0	0	0
3% modified starch	6.846	4.918	3.525	0	0	0

Appendix Table B18 Viable cell number of *L. reuteri* KUB-AC5 in 40°C dried pelleted feed with protective agents during at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
CMC	7.30E+07	2.08E+07	6.85E+06	5.65E+06	3.85E+06	1.11E+06
Sodium alginate	4.75E+07	1.33E+07	6.55E+06	3.45E+06	2.75E+06	3.07E+06
Milk powder	1.64E+08	1.19E+08	1.64E+07	1.33E+07	8.95E+06	6.20E+06
MSG	6.90E+07	3.75E+07	1.68E+07	9.05E+06	1.12E+07	1.11E+07
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
CMC	7.86	7.19	6.91	6.78	6.41	6.00
Sodium alginate	7.66	7.55	6.86	6.81	6.65	6.24
Milk powder	8.21	7.62	7.21	7.03	6.93	6.75
MSG	7.84	7.57	7.22	6.96	7.05	7.04

Appendix Table B19 Viable cell number of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed with protective agents during at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
CMC	1.09E+08	1.32E+07	2.85E+07	6.40E+06	2.12E+06	9.84E+05
Sodium alginate	9.55E+07	2.29E+07	1.30E+07	3.75E+06	5.35E+06	2.66E+06
Milk powder	1.41E+08	3.70E+07	9.35E+06	1.32E+07	6.90E+06	5.40E+06
MSG	6.10E+06	1.86E+06	9.30E+05	7.20E+05	6.15E+05	8.90E+05
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
CMC	8.03	7.12	7.45	6.81	6.33	5.99
Sodium alginate	7.97	7.36	7.11	6.57	6.73	6.42
Milk powder	8.15	7.57	6.97	7.12	6.83	6.73
MSG	6.79	6.27	5.97	5.85	5.79	5.95

Appendix Table B20 Viable cell number of *L. reuteri* KUB-AC5 in 40°C dried pelleted feed with protective agents during at storage 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)				
	0	10	30	60	90
CMC	7.30E+07	4.50E+05	2.71E+04	0	0
Sodium alginate	4.75E+07	3.20E+05	0.00E+00	0	0
Milk powder	1.64E+08	2.02E+06	8.55E+03	0	0
MSG	6.90E+07	7.25E+06	1.01E+04	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)				
	0	10	30	60	90
CMC	7.86	5.65	4.43	0	0
Sodium alginate	7.66	5.51	0.00	0	0
Milk powder	8.21	6.31	3.93	0	0
MSG	7.84	6.86	4.00	0	0

Appendix Table B21 Viable cell number of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed with protective agents during storage at 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)				
	0	10	30	60	90
CMC	1.09E+08	2.85E+06	1.58E+05	0	0
Sodium alginate	9.55E+07	1.35E+06	1.56E+03	0	0
Milk powder	1.41E+08	5.30E+06	3.10E+05	0	0
MSG	6.10E+06	4.70E+05	6.60E+04	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)				
	0	10	30	60	90
CMC	8.03	6.74	3.69	0	0
Sodium alginate	7.97	6.29	3.80	0	0
Milk powder	8.15	7.55	4.63	0	0
MSG	6.784	5.67	4.819	0	0

Appendix Table B22 Viable cell number of spray-dried *L. reuteri* KUB-AC5 in 40°C dried pelleted feed during storage at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
1% modified starch	3.10E+06	2.10E+06	1.74E+06	1.36E+06	1.20E+06	8.45E+05
2% modified starch	9.75E+06	7.85E+06	7.20E+06	6.50E+06	5.25E+06	5.00E+06
3% modified starch	3.15E+06	2.93E+06	2.39E+06	2.16E+06	1.23E+06	7.00E+05
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
1% modified starch	6.49	6.32	6.24	6.13	6.08	5.93
2% modified starch	6.99	6.89	6.86	6.81	6.72	6.70
3% modified starch	6.50	6.47	6.38	6.33	6.09	5.85

Appendix Table B23 Viable cell number of spray-dried *L. reuteri* KUB-AC5 in 40°C dried pelleted feed during storage at 4°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)					
	0	30	60	90	120	150
1% modified starch	1.10E+06	6.00E+05	8.60E+05	4.05E+05	4.50E+05	1.02E+05
2% modified starch	6.20E+07	4.45E+07	2.14E+07	1.66E+07	1.30E+07	1.71E+07
3% modified starch	4.80E+06	1.25E+06	1.15E+06	7.55E+05	6.45E+05	4.55E+05
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)					
	0	30	60	90	120	150
1% modified starch	6.04	5.78	5.93	5.61	5.65	5.00
2% modified starch	7.79	7.65	7.33	7.22	7.11	7.23
3% modified starch	6.68	6.10	6.06	5.88	5.81	5.66

Appendix Table B24 Viable cell number of spray-dried *L. reuteri* KUB-AC5 in 40°C dried pelleted during storage at 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)				
	0	10	30	60	90
1% modified starch	3.10E+06	3.30E+06	5.05E+04	0	0
2% modified starch	9.75E+07	7.15E+06	5.35E+05	0	0
3% modified starch	3.15E+06	2.73E+05	2.30E+04	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)				
	0	10	30	60	90
1% modified starch	6.49	5.85	4.73	0	0
2% modified starch	7.99	6.74	5.80	0	0
3% modified starch	6.50	5.91	4.12	0	0

Appendix Table B25 Viable cell number of *L. reuteri* KUB-AC5 in 50°C dried pelleted feed with protective agents during storage at 30°C

Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (cfu/g)				
	0	10	30	60	90
1% modified starch	3.10E+06	3.30E+06	5.05E+04	0	0
2% modified starch	6.20E+07	2.45E+07	5.35E+05	0	0
3% modified starch	4.80E+06	6.85E+05	5.25E+04	0	0
Pelleted feed with various treatment	Viable cell number of *L. reuteri* KUB-AC5 (log cfu/g)				
	0	10	30	60	90
1% modified starch	6.04	6.18	4.55	0	0
2% modified starch	7.79	7.39	5.73	0	0
3% modified starch	6.68	5.83	4.72	0	0

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