CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) group of bacteria are Gram-positive, nonsporulating, cocci or rods, prefer to grow under anaerobic conditions, but they can also grow in the presence of oxygen. They are protected from oxygen by-products (e.g. H_2O_2) because they have peroxidases. LAB ferment carbohydrates and produce lactic acid as their major end product including others, which contribute to the enormous environmental and health benefits (Khalid, 2011).

LAB are used for the production of fermented milk products like yoghurt and cheese, fermented vegetables such as sauerkraut, cucumber pickles, and olives and fermented fish and meat (Juodeikiene et al., 2012). LAB have an important role in food fermentation and preservation against pathogenic microorganism. This is due to the ability of LAB to produce organic acids such as lactic acid and acetic acid and also contribute in texture and flavor improvement and maintain the nutritional value of many milk and fermented food products (Gemechu, 2015). The main lactic acid bacteria associated with the milk and milk product fermentation is presented in Table 1. Fermented milk products contribute to human health through several mechanisms, for example *Lb. helveticus* produce peptide from casein milk protein which have beneficial activity for example, antihypertensive, immune modulator activity, and anticancer property as shown in Table 2 (Gemechu, 2015).

Table 1: The most important LAB associated with milk product fermentation (Gemechu, 2015).

Species/ subspecies	Their main uses in different milk products	References
Lactococcus		
Lc. Lactis subsp. Lactis	Mesophilic starter used for many cheese types, butter and butter milk.	Broome et al. (2003) and Wouters et al. (2002)
Lc. lactis subsp. Lactis biovar diacetylactis	Used in Gouda, Edam, sour cream and lactic butter and butter milk.	Wood (1997) and Leroy and De Vuyst (2004)
Lc. Lactis subsp. cremoris	Mesophilic starter used for many cheese types, butter and butter milk.	Weerkam et al. (1996)
Streptococcus *Sc. thermophilus*	Thermophilic starter used for yogurt and many cheese types' particularly hard and semihard high-cook cheeses.	Broome et al. (2003) and Beresford et al. (2001)
Lactobacillus		
Lb. acidophilus	Probiotic adjunct culture used in cheese and yogurt.	Briggiler-Marcó et al. (2007)
Lb. delbrueckii subsp. Bulgaricus	Thermophilic starter for yogurt and many cheese types, particularly hard and semihard high-cook cheeses.	Slaterry et al. (2010)
Lb. delbrueckii subsp.lactis	Used in fermented milks and high-cook cheese.	Broome et al.(2003) and Giraffa 2010
Lb. helveticus	Thermophilic starter for fermented milks and many cheese types particularly hard and semihard high-cook cheeses	Broome et al. (2003) and Griffiths and Tellez (2013)
Lb. casei	Probiotic milk and cheese ripening adjunct culture	Briggs (2003) and Kongo (2013)
Lb. plantarum	Cheese ripening adjunct culture.	Leroy and De Vuyst (2004)
Lb. rhamnosus	Probiotic adjunct culture used in cheese	Coppola et al. (2005)
Leuconostoc *Ln. mesenteroides subsp. cremoris*	Mesophilic culture used for Edam, Gouda, fresh cheese, lactic butter and sour cream.	Weerkam et al. (1996) and Slaterry et al. (2010)

Lb. =Lactobacillus; Lc. =Lactococcus; Ln.=Leuconostoc; Sc.=Streptococcus, subsp.= subspecies.

Table 2: Health benefits of fermented milk (Gemechu, 2015)

Table 2. Health benefits when milk is fermented.

Effect of fermentation	Changes in milk	Effect on health
Increase in lactic acid bacteria levels	Reduced lactose content in milk Reduced content of bad bacteria	No diarrhea and bloating Improved gut health Prevention of protection from bacterial vaginosis and fungal infections in women Ability to digest remaining lactose in the fermented milk and use as energy source
Breakdown to shorter chain proteins	Identification of casein peptides and whey peptides with functional properties	Easier digestion Some with antihypertensive effects Some with pain relief effects Some with immune enhancing properties Some with calcium binding bone building properties
Increased acidity	Sharpness of taste	Prevents harmful bacterial growth in milk

Source: http://whqlibdoc.who.int/publications/2003/9241591196.pdf and ftp://ftp.fao.org/docrep/fao/007/y5686e/y5686e00.pdf

The primary purpose of fermenting food using LAB is to achieve desirable change in sensory characteristic (flavor, aroma and texture) and also to prevent the growth of pathogenic microorganism. LAB are divided into two groups according to their fermentation pathways. The heterofermentative group produces organic acid and carbon dioxide as end product, while the homofermentative group produces principally lactic acid from carbohydrate fermentation as shown in Figure 1 (Caplice & Fitzgerald, 1999). LAB have a long history of application in fermented foods by addition of LAB as starter culture. They caused rapid acidification of the food during production of organic acid. In addition, LAB produces acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and several enzymes (Parada et al., 2007).

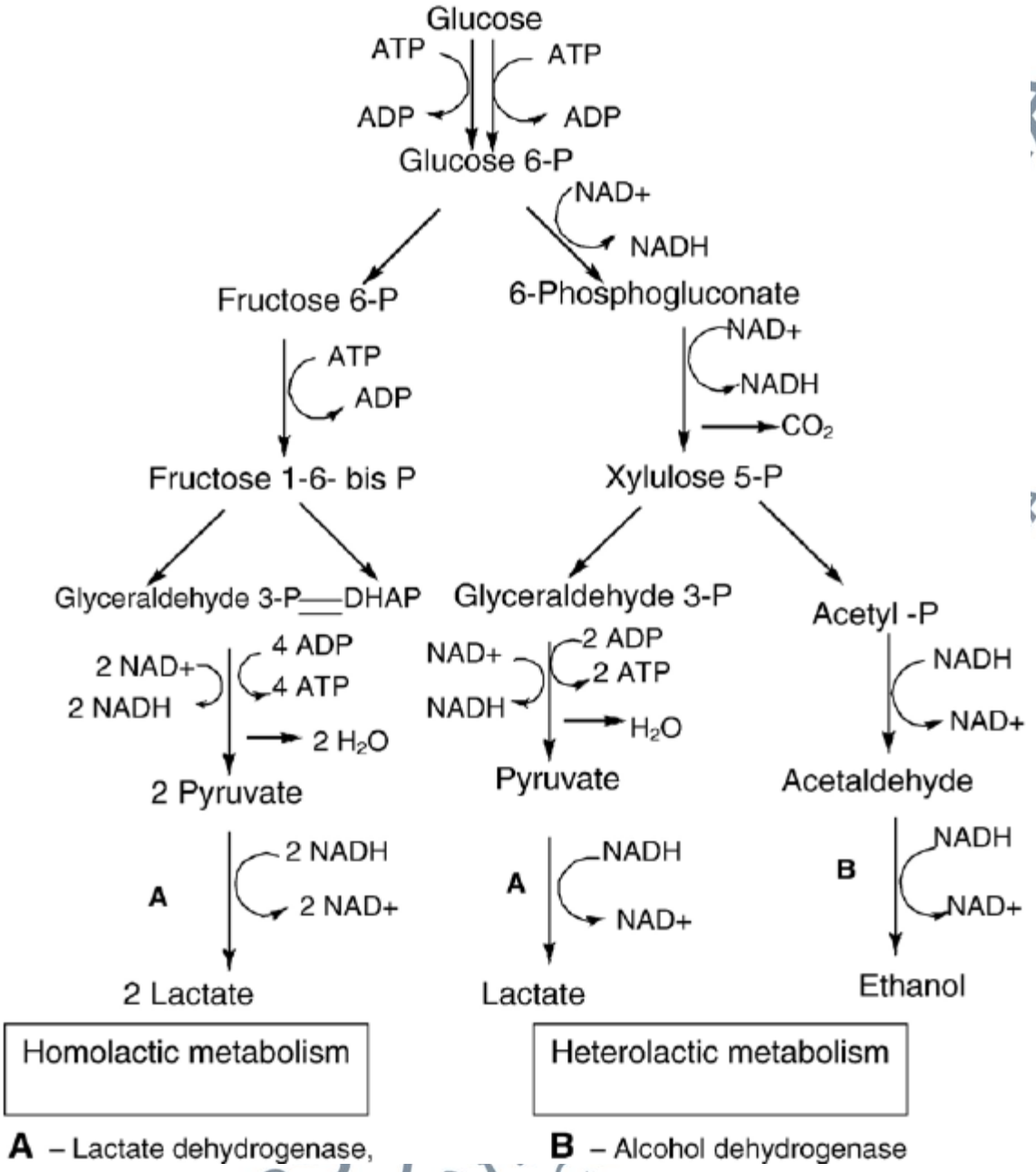

Figure 1: Generalized scheme for the fermentation of glucose in lactic acid bacteria

(Caplice & Fitzgerald, 1999)

2.2 Antimicrobial activity and anti-sporulation of LAB

LABs play an important role to inhibit the growth of pathogen and food borne diseases. LAB isolates from cheese had antibacterial effect against Gram positive bacteria *St. thermophilus* (Mezaini et al., 2009). Two methods were used to investigate the antimicrobial activity of lactic acid bacteria against indicator microorganism i.e. spot method and well diffusion assay, however, the spot method appear greater inhibition zone then the well diffusion assay (Cadirci & Citak, 2010). LAB showed inhibitory activity against *Salmonella Typhimurium*, *Listeria monocytogenes*, *B.cereus* and *E.coli* (Nordin et al, 2013). Different LAB (*Lb. plantarum* subsp**.** *plantarum, Pediococcus pentosaceus, Enterococcus mundtii*, *Weissella cibaria* and *Leuconostoc pseudomesenteroides* reported inhibitory activity against *Salmonella* Enterica ATCC 43971, *Micrococcus luteus* ATCC 4698 and *E. coli* ATCC 11775 (Li et al., 2015). Ammor et al. (2006) reported that the *Lactococcus garvieae, Vagococcus carniphilus, Enterococcus* sp. *Lb. sakei, E. faecium* showed inhibitory activity against *L. monocytogenes* and *E. coli*. *Lb. plantarum, Lb. bulgaricus, Lac. lactis* isolated from yoghurt, *Lb. delbrueckii* subsp. *bulgaricus, Lb. amylophilus, Lac. lactis* isolated from fufu, and *Leu. mesenteroides, Lb. plantarum, Lac. lactis* isolated from kunu showed inhibitory activity against *S. aureus, Shigella* spp., *Salmonella* Typhi and *E. coli* (Adejumo, 2014). Tropcheva et al. (2014) reported that *Lb. brevis* isolated from fermented curd/yogurt-like product "katak" showed antifungal activity against *Penicillium claviforme*, *Aspergillus awamori* and *A. niger*. LAB isolate from fermented guava juice show good inhibition activity against *A. oryzae* spore germination and mycelia growth (Muhialdin & Hassan, 2011).

Kıvanc et al. (2014) showed that LAB strains isolated from tarhana had inhibitory against spore germination of tested fungi *A. parasiticus, P. notatum, P. roqueforti, P. citrinum, P. griseofulvum, A. fumigatus, P. chrysogenum, A. oryzae, A. alternate*. LAB such as *Lb. sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7 and *P. pentoaceus* KTU05-8, KTU05-9 and KTU05-10 showed inhibitory activity against seed germination and seedling diseases (Suproniene et al., 2015). LAB isolated from Lithuanian sourdoughs such as *Lb. sakei* MI806, *P. pentosaceus* MI808, MI809 and MI810, *P. acidilactici* MI807, were reported able to produce bacteriocin-like inhibitory substance (BLIS) designated as sakacin 806, pediocin 808, 809, 810 and pediocin Ac807. These BLIS remained unaffected after enzymatic treatment with

commercially produced enzymes used in the bread making process namely hemicellulase, lipase, amyloglucosidase and amylase against the germination and growth of *B. subtilis* (Narbutaite et al., 2008).

LAB isolated from nonfat milk *Streptococcus lactis, S. thermophilus, Lb. acidophilus,* and *Lb. bulgaricus* showed inhibitory activity against growth of vegetative cells and germination of endospores of *B. cereus.* These LABs produced organic acid such as lactic acid, acetate, formate and lactate. Spore germination was not affected after 24 h, but was inhibited after 48 and 72 h incubation of the LAB. Acetate was moreinhibitory to vegetative cell while formate was more inhibitory against spore germination (Wong & Chen, 1988). The LAB cells and their neutralized cell-free supernatant reduce significantly the germination of fungal spores (*P. citrinum, A. niger* and *A. flavus*) (Adebayo & Aderiye, 2010). LAB do not only produce lactic and acetic acid, but also some strains of lactic acid bacteria produce other low molecular weight compounds with antifungal activities. One of these is phenyllactic acid (PLA) that showed inhibitory activity against sporulation fungal *A. niger, Cladosporium cladosporioides* and *P. roquefort* (Svanström et al., 2013).

Klewicka and Libudzisz (2004) reported that strains of *Lb. acidophilus* had inhibitory activity against *E. coli, Pseudomonas fluorescens, P. aeruginosa, S. aureus* and vegetative cells and spores germination of bacterial spores of *B. mycoides* and *B. subtilis* due to lactic and acetic acids and hydrogen peroxide, while low level accumulation of acetaldehyde and ethanol can play only a small supporting antibacterial role by this type of LAB.

Urinary tract infection (UTI) is a type of human diseases cause by antibiotic-resistant pathogenic bacteria. LAB including *Lb. acidophilus, Lb. paracasei, Lb. delbrueckii, Lb. casei, Lb. helveticus, Lb. brevis, Lb. salivarius, Lb. fermentum, Lb. rhamnosus, Lb. animalis,* and *Lb. plantarum* isolated from spoiled fruits and vegetables had antimicrobial activity against multi-drug-resistant uropathogens, viz. *C. albicans, P. aeruginosa, K. pneumoniae, E. fecalis,* and *E. coli* (Manzoor et al., 2016). Singh et al. (2016) reported that *Lb. acidophilus, Lb. fermentum, Lb. plantarum, Lb.casei* and *Lb. rhamnosus, Lb. acidophilus* isolated from raw milk, buttermilk, cucumber, pumpkin, curd, cheese, prebiotics infant formulas, tomato, carrot, banana, cabbage and apple, respectively showed moderate to strong inhibition against *S. aureus* and *Aspergillus* strains. The maximum zone of inhibition was 28 mm except against *E. coli.* These

LAB were resistant to lower concentrations of antibiotics but become susceptible at higher concentrations. These characteristics have potential to be used as a natural preservative and for probiotic applications**.** LAB cab be isolated using four different solid media namely, HJ (Hogg and Jago) media, KT (kiuru and Tybek) media, DO (Dougles) media and MRS (Mann Rogosa and Sharpe) media. Bactriocin produced by LAB with molecular weight (less than 14 kDa) as determine by SDS-PAGE can act against *E. coli* and *Kleibshella* sp. (Upendra et al., 2016)**.** The antimicrobial activity *Lb. casei, Lb. bulgaricus, S. thermophilus* and *Lb. bulgaricus* was highest in the cell suspension in MRS after 18 hrs incubation, however *S. thermophilus* with *L. bulgaricus* had the highest antibacterial against *S. aureus* with a zone inhibition of 10.5±0.35 mm and for *E. coli* 4±0 mm in contrast to the antimicrobial activity of a cell free extract against (Lekha Ravindran, 2016). This could be due to the amounts of acid produced and hydrogen peroxide in cell suspension in MRS (Gilliland & Speck, 1977).

LAB found in in milk, meat and fermented products, as well as in fermented vegetables and beverages inhibiting growth of pathogenic microorganism *E. coli, Pseudomonas* sp. *Salmonella* Typhimurium, *Salmonella* Para-typhimurium B, and *Clostidium* sp.) and, Gram-positive strains (*S. aureus, Streptococcus* sp., *L. ivanovii, B. megaterium* ID 07817 and *B. megaterium* ID 07818) due to the production of lactic and other organic acids is an important factor for the inhibition of growth of undesired microorganisms (Djadouni & Kihal, 2012). LAB also produce antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Yang et al, 2012).

Cizeikiene et al. (2013) reported that the presence of organic acids and bacteriocins-like inhibitory substances (BLIS) indicated that these LAB can be used in the food production as preservative due to their inhibition activity. LAB producing bacteriocin were isolated from Burkina Faso fermented milk. Strains of *Lb. fermentum, Pediococcus* spp., *Leu. mesenteroides* subsp. *meseteroides,* and *Lactococcus* exhibited antibacterial activity against *E. faecalis* 103907 CIP, *B. cereus* 13569 LMG, *S. aureus* ATCC 25293 and *E. coli* 105182 CIP using the agar drop diffusion test (Savadogo et al., 2004). Ammor et al. (2006) reported that *Vc. carniphilus, Lc. garvieae* LAB isolated from a small-scale facility producing traditional dry sausages had inhibitory activity against both *L. innocua* and *S. aureus* species due to ability of these strain to produce BLIS. Reis et al. (2012**)** reported that the LAB produce lactic acid, acetic acid, benzoic

acid, propionic acid, hydrogen peroxide, antifungal peptide, formic acid (De Keersmaecker et al., 2006) organic acids and reuterin (Gänzle et al., 2000). While LAB produced organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoine, carbon dioxide, reuterin, reutericyclin and bacteriocins (Šušković et al., 2010). Savadogo et al. (2006) reported that some strains of LAB produce antifungal compound such as s fatty acids, phenyllactic acid and bacteriocins.

2.3 Antimicrobial compound produce by LAB

LAB can produces variety of antimicrobial compounds such as lactic acid, acetic acid, ethanol, formic acid, fatty acids, hydrogen peroxide and bacteriocins (Upendra et al., 2016).

2.3.1 Organic acid

Šušković et al. (2010) reported that the organic acids such as lactic and acetic acid produce by LAB have ability to prevent growth of pathogenic microorganism due this reason the LAB can be used in food industry as starter cultures and probiotic bacteria. LAB can produce low-molecular mass antimicrobial compound such as 2-pyrrolidone-5-carboxylic acid (PCA), also known as pyroglutamic acid (Yang, 2000). LAB such as *Lb. plantarum* IMAU 10124, *Lactobacillus* sp. SK007 and *Lb. lactis* NCIM 5449 can produce 3-phenyllactic acid (PhLA) by fermentation and biotransformation (Ström et al., 2002). Piard an Desmazeaud (1991) reported that LAB produce organic acids (lactic, acetic and formic). Lactococci produce lactic acid, acetic acid, succinic acid inhibited the growth of *E. coli, P. aeruginosa* and *S. aureus*. Lactic acid was in large amounts and phenyllactic acid was produced only by *Pediococcus* sp. (Hladíková et al., 2012). Zalán et al. (2010) reported that all strains of LAB *Lb. rhamnosus, Lb. plantarum*, *Lb. paracasei*, *Lb. casei* and *Lb. curvatus*, except *Lb. plantarum* 01 produced lactic acid in skimmed milk, MRS broth and Jerusalem artichoke (JA) medium. Production of acetic acid was influenced by strains and growth media. The LAB strains produced acetic acid in MRS broth except *Lb. curvatus* 2768 and *Lb. casei* Shirota. Acetic

acid was not detected present in JA broth except for *Lb. paracasei* SF1 and in skimmed milk except for *Lb. casei* 2750, *Lb.curvatus* 2768, *Lb. curvatus* 2775 and *Lb. casei* Shirota.

2.3.2 Hydrogen peroxide

Hydrogen peroxide produced by LAB inhibits the growth of pathogens (Dahiya & Speck, 1968) in the presence of oxygen (Dalié et al., 2010). Kang et al. (2005) reported that H_2O_2 with lactic acid produced by LAB kill foodborne pathogen such as *E. coli* O157:H7, *S.* Enteritidis, and *L. monocytogenes.* The antimicrobial activity of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell and destroy the basic molecular structures of cell protein. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability, also H_2O_2 can produce bactericidal free radical such as superoxide (O_2^-) and hydroxyl (OH^-) radicals which can damage DNA (Amenu, 2013). Wakil and Osamwonyi (2012) reported that H_2O_2 produce by *Lb. plantarum, Lb. fermentum, Luec. meseteriodies, Lb. jensenii, Lb. brevis, P. acidilactici* and *Lactobacillus* spp. showed inhibition activity against *B. cereus, E. coli. S. aureus, B. licheniformis, P. flourescens, P. aeruginosa, Proteus* spp., *Salmonella* spp., *Serratia* spp. and *P. syringae. Lb. acidophilus* produces 56.0 to 72.0 µg/mL of this compound in milk can efficiently inhibit the growth of *S. aureus*, while higher concentrations, e.g. 50.0 µg/mL and more, can prolong the lag phase of *Pseudomonas* species (Klewicka & Libudzisz, 2004). Falagas et al. (2007) reported that lactobacilli inhibit the growth of bacteria causing bacterial vaginosis by producing H_2O_2.

LAB produce hydrogen peroxide due to the absence of catalase in these microorganisms that causes the accumulation of hydrogen peroxide (H_2O_2) which inhibits bacteria and fungi (Fadahunsi, 2015). Ito et al. (2003) reported that H_2O_2 accumulated by lactic acid bacteria can effect in reducing the viable cell count of food-borne pathogens such as *Ent. faecalis, Ent. faecium, Yersinia enterocolitica,* enterotoxigenic *E. coli, S. aureus, L. ivanovii* and *Aeromonas hydrophila*. *Lb. plantarum* 2142 in MRS broth produced the highest concentration of hydrogen peroxide (7.5 g/107 CFU) cause inhibitory effect against the food spoilage bacteria using the agar diffusion method (Zalán et al., 2005).

2.3.3 Bacteriocins

Bacteriocins are proteinaceous antibacterial compounds, both Gram negative and Gram positive bacteria produce this compound (Savadogo et al., 2006). Bacteriocins play an important role in food application due to ability control of food spoilage and pathogenic food-borne microorganisms (Musikasang et al., 2012) It gives a benefit in reducing the use of chemical preservatives (Udhayashree et al., 2012). Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria specially LAB (Gálvez et al., 2007). Bacteriocins are digested by proteases enzyme in the human digestive system without change in their activity compared with therapeutic antibiotics (Parada et al., 2007).

Bacteriocins are initially isolated from Gram-negative bacteria A colicin from *E. coli* and other Gram-negative bacteria produce colicin-like proteins including klebicins of *Klebsiella pneumonia*, marcescins of *Serratia marcescens*, alveicins of *Hafnia alvei*, cloacins of *Enterobacter cloacae* and pyocins of *Pseudomonads.* Due to ed antimicrobial activity for the bacteriocins of Gram-negative bacteria, they are not use in the food industry (Güllüce et al., 2013) compare to bacteriocins produced by Gram-positive bacteria (Cleveland et al., 2001).

High bacteriocin production by *Lb. ivanovii* was obtained with MRS agar incubated at 30ºC after 24 h. The absence of specific nutrients were required for bacteriocin production recorded in brain heart infusion (BHI) and M17 agar (Djadouni & Kihal, 2012). However**,** De Kwaadsteniet et al. (2005) elucidated that highest activity of bacteriocin produced by *E. mundtii* ST15 was recorded after 14 h of growth in MRS broth at 30ºC, while the lowest activity was recorded in M17 broth and BHI broth. It was reported that MRS broth is the best medium for the LAB to produce bacteriocin due to presence of undefined compounds in the complex nitrogen source in the MRS broth (Leroy & De Vuyst, 2001). Research carried out by Biswas et al. (1991) observed that the production of pediocin AcH by *P. acidilactici* H in large quantities can be achieved in TGE broth (Tryptone Glucose Extract broth) within 16 to 18 h at 30 to 37°C (final pH, 3.6 to 3.7). Tryptone-yeast extract-tween (TYT10, TYT11 and TYT30) were designed to maximize bacteriocin production while minimizing the amount of peptides in the medium by *Ent. faecium* DPC 1146 (enterocin 1146), *Lac. lactis* subsp. *lactis* biovar diacetylactis DPC 3286 (lactocin D) and *Lac. lactis* subsp. *cremoris* LMG2130 (lactococcin A) (Parente & Hill, 1992).

Lactococcus lactis subsp. *lactis* A164 produced at least 4-fold greater bacteriocin in M17 broth supplemented with lactose than other carbon sources at 30 ^{o}C (Cheigh et al., 2002), optimal bacteriocin production was recorded in MRS after 24 h with an initial pH of 6.5, 6.0 and 5.5. Low levels of bacteriocin activity were recorded in MRS broth with an initial pH of 5.0 and 4.5 (Djadouni & Kihal, 2012).

There are several proposed bacteriocin classifications and divided into 3 or 4 classes (da Silva Santos't, 2014).

(i) Lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose biologically inactive prepeptides are subjected to extensive post-translational modification.

(ii) Small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocins like or *Listeria*-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb) and circular bacteriocins (class IIc).

(iii) Bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III). Some authors also proposed (class iv) bacteriocins that require non-proteinaceous moieties (lipid, carbohydrate) for their activity.

Nisin is the first used for food production in 1931 and first received approval by Food and Drug Administration (FDA) to be used in pasteurized processed cheese in 1988 (Karthikeyan & Santosh, 2009). Mezaini et al. (2010) reported that bacteriocin, produced by *S. thermophilus* T2, was stable at pH range 4 to 8; showed antibacterial effect against Gram positive and Gram negativebacteria. Simova et al. (2009) reported that LAB strain *Lb. casei* ssp. *rhamnosus* (PC5), *Lac. lactis* ssp. *lactis* (BCM5, BK15), *Lb. plantarum* (BR12), *Lb delbrueckii* ssp. *bulgaricus* (BB18), *Ent. Faecium* (MH3) and *Lb. casei* ssp. *casei* (BCZ2) isolated from authentic Bulgarian dairy products had ability to produce bactriocin capable to inhibit growth of pathogenic microorganism. *Lactobacillus* strains produced bacteriocin had inhibitory activity against *Lb. gasseri, Lb. acidophilus, Gardnerella vaginalis* ATCC 14018 and *P. aeroginosa* ATCC 10145 (Karaoğlu et al., 2003). Hashium et al. (2010) reported that bactriocin produce *Lb. acidophilus* showed inhibitory activity against Gram-positive and Gram-negative bacteria namely, *S. albus* (MTCC7407), *S. aureus* (MTCC7405), *P.*

aeruginosa (MTCC4676), *B. subtilis* (MTCC3053), *Str.. pyogenes* (MTCC 1926), *E. coli* (MTCC1674), and *Micrococcus luteus* (MTCC4428). Bacteriocinogenic LAB strains may play in the food industry as starter cultures, in sourdough (to increase competitiveness), in fermented sausage (anti-listerial effect), and in cheese (anti-listerial and anti-clostridial effects) (De Vuyst & Leroy, 2007). Bactriocin producing LAB strain isolated from raw milk had inhibitory activity against Gram positive *Staphylococcus, Bacillus* and *L. monocytogenes* and Gram negative bacteria including *P. aeruginosa* and *E. coli* (Daba & Saidi, 2015). Bacteriocin produced by *Lb. plantarum* LBP01 had inhibitory activity against *E. coli* (Saidi et al., 2011). Mohammed and Ijah (2013) reported that LAB isolated from yoghurt, wara (cheese) and nono (fermented milk) were identified as *Str. thermophilus, Lac. lactis, Lb. acidophilus, Lb. lactis, P. halophilus, P. cerevisiae, Str. cremoris* and *Lb. bulgaricus* showed inhibitory activity against *Salmonella* spp. and *Bacillus* spp. due to ability of these LAB stain to produce antimicrobial compound such as bactriocin.

Bacteriocins produce by LAB such as nisin, Enterocin AS-48, Lacticin 3147, Bificin C6165, Plantaricin TF711, Thurincin H can prevent the outgrowth of spore in food. Egan et al. (2016) reported that LAB produce a small number of bacteriocins that showed activity against spore germination of different bacteria such as Nisin against A. *acidoterrestris, B. myloliquefaciens, B. cereus, B. subtilis* and *C. beijerinckii*; Enterocin AS-48 against *A. acidoterrestris, B. cereus, B. licheniformis,* and *G. stearothermophilus*; Enterocin AS-48 against *A. acidoterrestris, B. cereus, B. licheniformis* and *G. stearothermophilus*; Plantaricin TF711 against *B. cereus* and *C. sporogenes.* De Carvalho et al. (2007) reported that Bovicin HC5 bactriocin produced by LAB inhibited and reduced the outgrowth of spores from *B. cereus* and *B. thuringiensis* isolated from spoiled mango pulp.

Various physicochemical factors seemed to affect bacteriocin production as well as its activity; maximum activity was noted at pH 5, temperature 40°C and 0.9% NaCl (Karthikeyan & Santosh, 2009). Treatment of bacrtiocin with all the proteolytic enzymes, catalase, dextranase, phospholipase C, lysozyme, lipase, amylase cause loss of their activity while, mitomycin and UV light did not affect the activity of bacteriocin (Ogunbanwo et al., 2003). Tomé et al. (2009)

reported that loss of anti-bacterial activity of the bactriocin produce by *Lb. curvatus, Lb. delbrueckii, Lb. fermentum, Ent. faecium,* and *P. acidilactici* due to treatment with trypsin, protease E, and proteinase K permits peptides degraded in the intestinal tract without affecting the intestinal flora. However, their antimicrobial activities were not completely inactivated by trypsin and pepsin (Batdorj et al., 2006).

Ribeiro et al. (2008) reported that the antimicrobial activity of cell free supernatant (CFS) was not influenced with 2% NaCl. Some isolates were sensitive to 4% NaCl, but three isolates identified as *E. faecalis* (L2B21K3, L3B1K3 and L3A21K6) continue to produce bacteriocins at 10% NaCl, Most of denaturing agents, with the exception of SDS, did not affect antimicrobial activity of bacteriocins. In contrast, treatments with organic solvents result in decrease or total loss of antimicrobial activity for some isolates. The antimicrobial activity of the supernatant LAB *Lb. paracasei* ssp. *paracasei, Lac. lactis* ssp. *lactis, Lb. brevis* and *Lb. pentosus* remain unaffected after treatment with chloroform against *E. coli* and *Salmonella* spp. However, increase in the inhibitory activity was observed after treatment by detergents Triton-X 100 and EDTA (Tenea & Yépez, 2016).

2.3.4. Reuterin

Lactobacillus reuteri, produced reuterin from glycerol by heterofermentative, a normal inhabitant of the human intestine and is a broad-spectrum antimicrobial agent against Gram positive and Gram negative bacteria (Cleusix et al., 2007; Biane et al.,2011). Liu and Yu (2015) reported that Reuterin is a compound that contains the monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA). Reuterin formed by metabolism of glycerol and change in colour because of the acrolein formed by dehydration of b-hydroxypropionaldehyde (Rodriguez et al., 2003). Fermentation pathway for the production of reuterin in *Lb. reuterin* is shown in Figure 2 (Liang & Sung, 2001). The mechanism of action by which reuterin induces oxidative stress in cells, most likely by modifying thiol groups in proteins and small molecules (Rodriguez et al., 2003).

Reuterin produce by *Lb. reuterni* inhibited the growth of three *L. monocytogenes* starins (Montiel et al., 2014) and the addition of purified reuterin might be used to improve the safety

and extend the shelf-life. Reuterin is produced during anaerobic fermentation of glycerol by *Lactobacillus reuteri* strain showed inhibitory activity against *L. monocytogenes, E. coli, S.* Typhimurium, *P. aerogenosa, K. penomoniae* and *S. aureus* (El-Ziney et al., 1999).

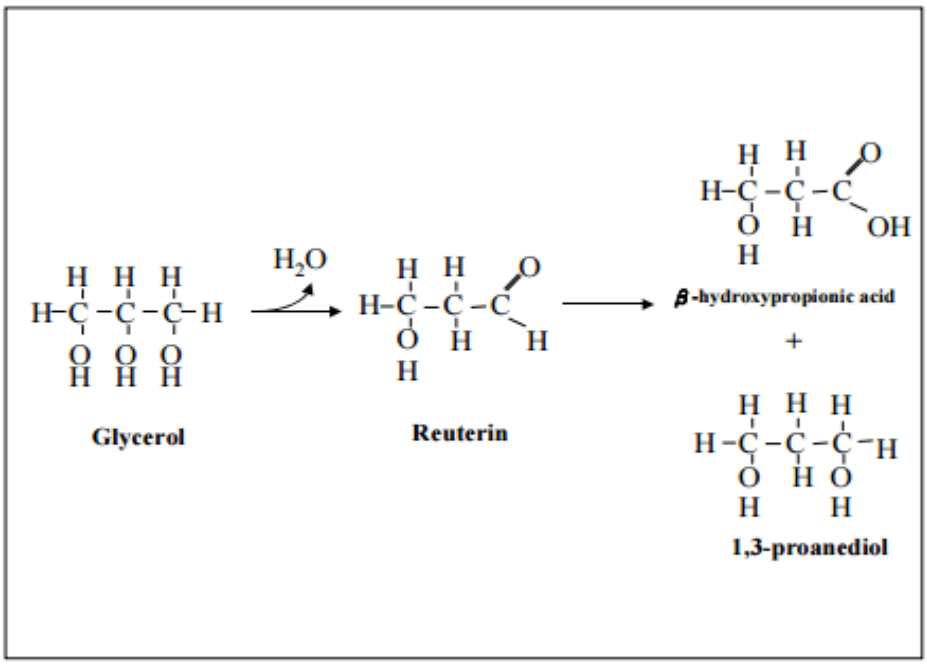

Figure 2: Fermentation pathway for the production of reuterin in *Lactobacillus reuterin*

(Liang & Sung, 2001).

Reuterin is active toward enteropathogens, yeasts, fungi, protozoa and viruses, but its effect on commensal intestinal bacteria is unknown (Cleusix et al., 2007). *Lb. reuteri* DSM 17938 was successfully incorporated into a fresh cheese with high viability during the storage period, due to ability of this strain to produce reuterin and act against *E. coli* O157:H7 (EHEC) and *S. aureus* (Karlsson, 2013). *L. reuteri* have been reported to produce antibacterial compounds, bacteriocin and reuterin respectively, against a food-borne pathogen *C. jejuni* (Petsuriyawong & Khunajakr, 2010), *E. coli* and *L. moncoocytogenes* (Khunajakr et al., 2008). Some reports mentioned that other LAB can synthesis reuterin such as *Lb. brevis* and one strain of *Lb. buchneri* (Schütz & Radler, 1984), *Lb. coryniformis* (Nakanishi et al., 2002).

2.4 Mechanism of the antimicrobial activity of compound produces by LAB

Traditionally, microbial growth inhibition by organic acids was explained by the ability of these acids to pass across the cell membrane (Van Immerseel et al., 2006). Organic acid such as lactic and acetic acid produce during sugar fermentation lead to reduction in pH play an important role for the inhibition growth of undesired microorganisms includes pathogenic bacteria and food born disease, however, low pH makes organic acids lipossoluble, allowing them to penetrate the cell membrane and destroy the cytoplasm of pathogens (Parada et al., 2007). Dibner and Buttin (2002) reported that organic acids have an effect on pancreatic and bile secretion that is mediated by their ability to diffuse into cells when in the undissociated form and then to dissociate in response to the higher pH of the cell cytoplasm, due to the passage of undissociated acid molecules across the cell membrane. Once inside the cell, the higher pH of the cytoplasm will lead to dissociation of the acid. This will generate an accumulation of the anion of the acid together with protons and consequently a decrease of the intracellular pH (Ström, 2005), with eventual disruption of pH homeostasis and cellular metabolism (Lu et al., 2011). According to Figure 3, there are four broad-acting mechanisms that inhibit the bacterial cell (Desriac et al., 2013):

i. Acid stress disrupts cell regulation on a general level.
ii. Bacteria spend energy to maintain pH, by pumping out acid.
iii. Bacteria change their metabolism to produce alkaline metabolites.
iv. Acid stress generates free radicals that damage all cellular mechanisms.

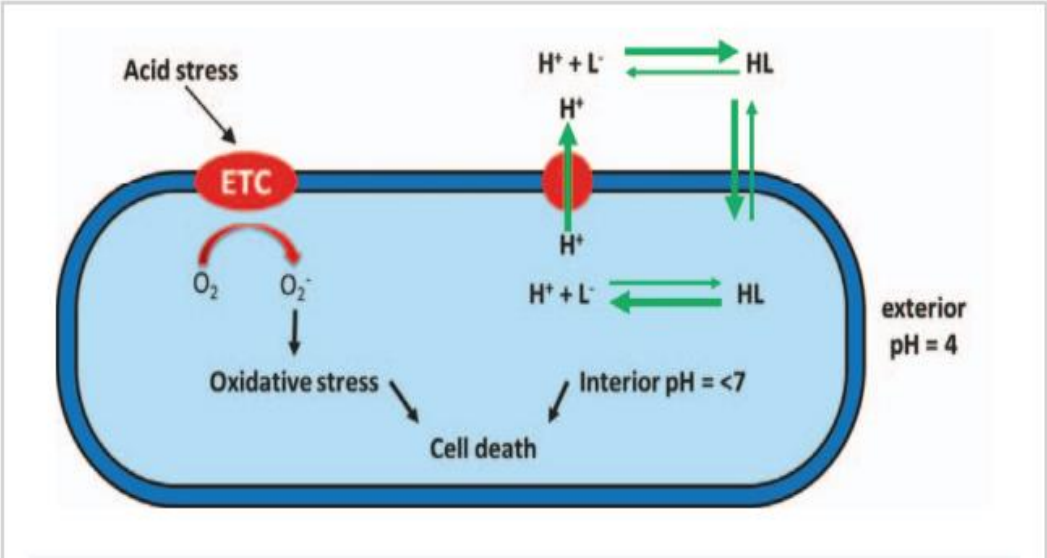

Figure 3**:** Schematic of main mechanisms by which Lactic Acid kills bacteria (Desriac et al., 2013).

In addition, the LAB can produce antimicrobial compound such as hydrogen peroxide that had inhibitory activity against undesirable microorganism. The mechanism of H_2O_2 cytotoxicity to bacteria is not yet clear because they are different structure of Gram-positive and Gram-negative bacteria in the outer membrane and cell wall (Brudzynski, 2006). Fadahunsi (2015) reported that the antimicrobial activity of hydrogen peroxide can be related to its oxidizing effect on the cell membrane of the pathogens and destroying of the cellular protein. The antimicrobial effect of H_2O_2 is due to oxidation of sulfhydryl groups lead to denaturing of a enzymes, and increased membrane permeability that form peroxidation of membrane lipids (H_2O_2 may be as a precursor for the production of bactericidal free radical such as superoxide (O_2 -) and hydroxyl (OH-) radicals which can damage DNA (Amenu, 2013).

The mode action of bactriocin however, is by different mechanisms such as inhibiting cell wall synthesis, permeabilizing the target cell membrane, or by obstruction RNase or DNase activity (Cleveland et al., 2001), alteration of enzymatic activity, inhibition of spore germination and inactivation of anionic carriers through the formation of selective and non-selective pores (Parada et al., 2007). LAB bacteriocins create pore on the target cell membrane that cause destruction of cytoplasm (Jack et al., 1995).

2.5 *Bacillus* spp.

Bacillus species are Gram-positive, facultative anaerobic, endospore-forming (Amin et al., 2015), have the ability to produce endospores that allow *Bacillus* to resist undesirable environment that occurring in food processing (Baruzzi et al., 2011). *Bacillus* spp. has ability to cause a human infection such as superficial wound or skin infection, closed space infections such as endophthalmities, severe systemic disease such as pneumonia (Weber et al., 1989). *Bacillus* spp. are associated with environmental reservoirs such as contaminated air filtration systems, ventilator equipment, dressings, gloves, hands of healthy staff, intravenous catheters, alcohol-based handwash solutions, specimen collection tubes, blood culture media and linens (Ozkocaman et al., 2006).

Bacillus cereus isolated from soil and growing plants are easyly spread to food and cause an emetic or a diarrhoeal related type of food-associated illness. There are three toxins that have been implicated of the diarrhoeal disease cytotoxins haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (Arnesen et al., 2008). Stenfors et al. (2002) reported that *B. cereus* produces one emetic toxin and at least three enterotoxins associated with the diarrhoeal type of food poisoning. The food poisoning caused by *B. cereus* is due to the ingesting heat-stable enterotoxins (Schneider et al., 2004). *Bacillus cereus* prefers different condition for growth, three L- amino acids (threonine, leucine, valine), temperature range for growth at 4 to 55°C, minimum water activity (a_w) for growth is 0.93 and the pH range is 4.3 - 9.3 (Jääskeläinen, 2008).

Bacillus strains including *B. subtilis* isolated from wheat flour samples had ability to produce rope in baked bread (Yibar et al., 2012). Other study reported that *B. subtilis* was the most abundant species in both white and whole meal breads (Erem et al., 2009). *B. subtilis* consists of three subspecies, which include *Bacillus subtilis* subsp. *subtilis*, subsp. *spizizenii* and subsp. *inaquosorum* (Alina et al., 2015). The critical *B. subtilis* subsp. *spizizenii* ATCC 6633 levels cause ropiness in baked bread, by decomposing proteins and carbohydrates of bread crumb, turning the bread pulp into a sticky, slimy, and foul smelling mass. This cause unpleasant sweet, musty smell of rotting pineapples (Vaičiulytė-funk et al., 2015). Optimum growth temperature for *B. subtilis* is 30-37 °C, with a minimum temperature of 18 °C and a maximum of

43 °C (Cook, 1996), and the pH range from between 6.5 to 7 (Younis et al., 2010), water activity (measure of available water) greater than 0.94 (Gibbs & Gekas, 1998).

2.5.1 Spore germination of *Bacillus* spp.

The *Bacillus* spp. can form spores when exposed to harsh conditions; these spore are formed within the mother cell (Setlow, 2014). The process of spore formation called sporulation cycle (Figure 4) is divided into seven different stages. The vegetative cells is in stage 0 undergo cell division to form a small forespore which develops into the spore and large mother cell (Pandey, 2014). *Bacillus* spp. spore consist of multilayer structure in which each layer plays an important role in protecting the spore against adverse conditions give the bacteria ability to survive for millions of years (Nguyen Thi Minh et al., 2010). Spores consist of a core, inner membrane, cortex, outer membrane, coat and exosporium (Figure 5) (de Vries, 2006).

Food poisoning spore-formers include *B. cereus, B. subtilis and B. licheniformis* (Brown, 2000). Contamination with spore causes serious food poisoning and it is very difficult to kill them in the food products because of their significant resistance (Yamazaki et al., 1997), *Bacillus* species produce endospores resist heat, radiation, and chemical treatments. The external layer of the spores produced by Bacillus species is called anexosporium, composed of a number of different proteins such as glycoproteins that play a major role in the spore's attachment to surfaces (Soni et al., 2016).

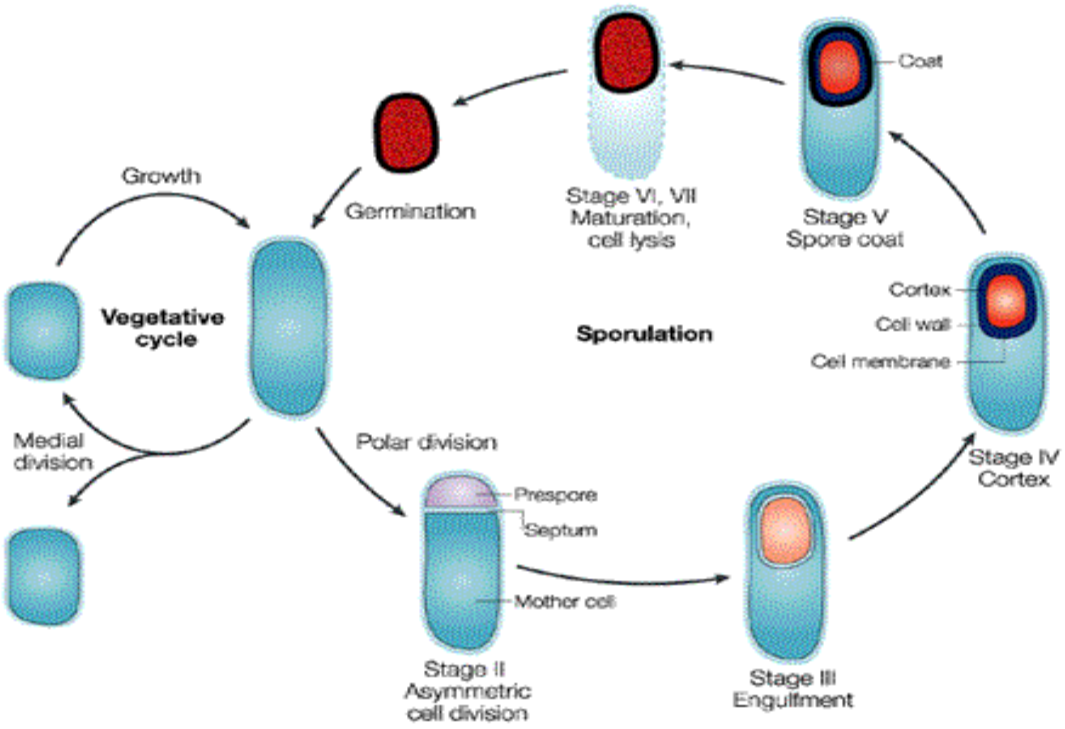

Figure 4: The sporulation cycle of spore-forming bacteria (Errington, 2003)

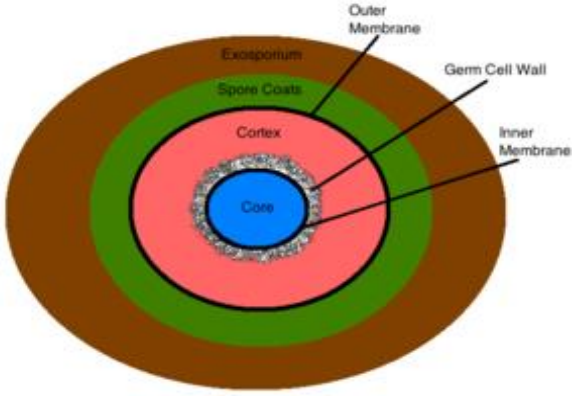

Figure 5: Schematic representation of the internal structure of a bacterial spore (Pandey, 2014)

Silva et al. (2013) reported that spores inactivation can be achieved by combined hydrostatic pressure and heat treatments, used pressure (300-500 MPa), temperature (30-50ºC), and pressure-holding time (10-30 min). Chemical disinfectants like calcium hypochlorite, free available chlorine, hydrogen peroxide inactivate *B. cereus*. Peracetic acid, cupric ascorbate, sodium hypochlorite can also inactivate *B. subtilis* spores (Sagripanti & Bonifacino, 1996). Smelt (1998) reported that the bacterial spore inactivation can be achieved by using Ultrahigh Pressure (UHP) up >1000 MPA, however, bacterial spore be stimulated to germinate by pressures of 50±300 MPa. DNA of *Bacillus* spp. spore damage by using some chemicals (e.g. nitrous acid, formaldehyde). Setlow (2006) and other research showed that High hydrostatic pressure HHP (~650 MPa and 50°C) combination with antimicrobials compound (lactoperoxidase or lysozyme and nisin) holds potential to inactivate *Bacillus* spores (Sarker et al., 2015).

Chemical disinfectants like calcium hypochlorite, free available chlorine, hydrogen peroxide inactivate *B. cereus* spores. Peracetic acid, cupric ascorbate, sodium hypochlorite can also inactivate *B. subtilis* spores (Sagripanti & Bonifacino, 1996). Bacterial spores can be removed by using filters with pore size ratings of 0.45µm for bacterial control in the soft drink manufacturing process, improves soft drink quality without exposing the flavor components to heat degradation (Sakuraoka & Madsen, 2001). DNA of *Bacillus* spp. spore are damaged by using some chemicals for example, nitrous acid, formaldehyde (Setlow, 2006).

2.5.2 Food spoilage by *Bacillus* spp.

Food spoilage is a complex reaction or sensory change (tactile, visual, olfactory or flavour) of foods due to insect damage, physical damage, indigenous enzyme activity or by microbial infections (Gram et al., 2002; Rawat, 2015). Microbial spoilage is caused by enzymes most of which are intracellular and some are extracellular (amylases, proteases and lipases) (Das et al., 2014).

Bacillus cereus and *B. subtilis* are common food spoilage, found in different types of raw food such as rice, meat, vegetables, raw milk, dairy products as well as cooked dishes, and cause food born disease emetic and diarrheal syndromes (Organji et al., 2015; Wogu & Ofuase, 2014).

Gopal et al. (2015) reported that species other than *B. cereus* and *B. subtilis* have confirmed the production of heat-labile toxins including *B. circulans, B. lentus, B. licheniformis, B. pumilus, B. amyloliquefaciens, B. licheniformis , B. pumilus, B. myloliquefaciens, B. mojavensis, B. simplex, B. firmus, B. megaterium, B. circulans* and *B. lentus.*

Bacillus spp. and *Clostridium* spp. are sporeforming bacteria that are present in foods are important due to the ability of the spore to resist heat, freezing, chemicals, and other adverse environments during processing and preparation of food (Cousin & Layfayette, 1989), Spores of *Bacillus* genus are prevalent in soil and the gut of insects and animals , as a result, so it's easy to be found in all kinds of food, cause food borne illness after consumption, however this spore can protect themselves against undesirable condition by phenotypic variability (Stecchini et al., 2013). Rodríguez-Lozano et al. (2010) reported that *B. pumilus, B. licheniformis, B. subtilis,* and *B. megaterium* associated with spoilage of food products at low 3.9, however, some *Bacillus* spp. have the ability to increase pH that allow the germination of any *C. botulinum* spores present in the product .

Beside *C. botulinum*. *B. cereus* one of the microorganisms Creates problems of public health present in canned with heat resistance less than that of *C. botulinum* (Bradshaw et al., 1975). Margosch et al., (2004) Reported that *C. botulinum* TMW 2.357 exhibited a greater resistance to pressure and temperature than *Bacillus* spp. spores (*B. cereus, B. subtilis, B. licheniformis, B. smithii, B. amyloliquefaciens*), except *B. amyloliquefaciens.*