UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF BACTERIOCIN PRODUCED BY Lactococcus lactis subsp. lactis S20 ISOLATED FROM CHINESE SAUERKRAUT AND ITS ANTILISTERIAL ACTIVITY IN MILK

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By

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CHARACTERIZATION OF BACTERIOCIN PRODUCED BY *Lactococcus lactis* subsp. *lactis* S20 ISOLATED FROM CHINESE SAUERKRAUT AND ITS ANTILISTERIAL ACTIVITY IN MILK

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February 2016

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Chemical preservatives might cause negative side-effects to public health. Much research have been done for searching natural preservatives to cope with the heightened public awareness. Therefore, the objective of this study is to characterize the bacteriocin isolated from traditional pickled vegetable and to evaluate its antilisterial activity in milk. Among the four traditional pickled vegetables studied, only Chinese sauerkrauts (*suan cai*) showed the presence of LAB with an average LAB count of 6.73 × 10^6 CFU/g. *Lactococcus lactis* subsp. *lactis* S20, a bacteriocinogenic lactic acid bacterium, was isolated from Chinese sauerkraut. This strain produced the highest bacteriocin activity (1280 AU/ml) against *Listeria monocytogenes* ATCC 19115 after 20 h of culture at 30°C in MRS broth, although results in this study demonstrate that TSB was a suitable alternative bacteriocin production medium. The quantification of bacteriocin activity was determined by using microtiter plate assay. *L. lactis* subsp. *lactis* S20 displayed the ability to ferment sucrose and xylose which are uncommon to *L. lactis*. Bacteriocin S20 exhibited inhibitory activity against most Gram-positive food pathogens but did not inhibit the growth of Gram-negative bacteria. Results of the stability tests indicate that bacteriocin S20 was resistant to trypsin, amylase, and lipase but sensitive to proteinase K, α-chymotrypsin, and pepsin. This bacteriocin was stable over a wide range of pH (pH 3-9) and at temperatures of up to 70°C for 15 min. The bacteriocin activity of bacteriocin S20 dropped to 40 AU/ml after autoclaving at 121°C for 15 min. Taken together, the results from the stability tests and molecular studies suggest that bacteriocin S20 is closely related to nisin A. The extraction of bacteriocin S20 was conducted by using the cell-adsorption method. Based on the bacteriocin adsorption study, pH 6 and pH 7 were the optimal pH for the adsorption of bacteriocin S20. The cells of the sensitive strain showed a higher adsorption of bacteriocin S20 than did the producer strain. Bacteriocin adsorption in the general medium TSB was relatively higher than that in MRS. Conversely, higher recovery yield and fold of purification were observed in bacteriocin S20 extracted from MRS. Bacteriocin S20 has shown to effectively control *Listeria* cells in milk system (UHT milk). After initially reduced by the bacteriocin S20, the *Listeria* counts remained almost constant throughout the 14 days of storage. Results also revealed that *Listeria* count of milk samples with 320 AU/ml bacteriocin S20 had little or no significant difference (p>0.05) to that of 160 AU/ml. In addition, bacteriocin S20 showed an effective antilisterial activity in skim milk containing <12% (w/v) milk solids-not-fat (MSNF). MSNF might interact with bacteriocin S20 and affect its antimicrobial activity in milk. In conclusion, bacteriocin S20 that have been successfully isolated from Chinese sauerkraut have a potential to be used as antilisterial in food.

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PENCIRIAN BAKTERIOSIN YANG DIHASILKAN OLEH *Lactococcus lactis* subsp. *lactis* S20 DIPENCILKAN DARIPADA SAUERKRAUT CINA DAN AKTIVITI ANTILISTERIAL DALAM SUSU

Oleh

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Bahan pengawet kimia berkemungkinan memberikan kesan sampingan negatif kepada kesihatan awam. Banyak penyelidikan telah dijalankan untuk mencari bahan pengawet semula jadi. Oleh yang demikian, objektif bagi kajian ini adalah untuk mencirikan bakteriosin yang dipencilkan daripada sayur jeruk tradisional dan seterusnya menilai aktiviti antilisterial dalam susu. Antara empat sayur-sayuran jeruk tradisional, hanya sauerkrauts Cina (*suan cai*) menunjukkan kehadiran LAB dengan kiraan 6.73 × 10^6 CFU/g LAB. *Lactococcus lactis* subsp. *lactis* S20, bakteria laktik asid yang menghasilkan bakteriosin, telah dipencilkan daripada sauerkraut Cina. Strain ini didapati mempunyai aktiviti bakteriosin yang paling tinggi (1280 AU/ml) terhadap *Listeria monocytogenes* ATCC 19115 selepas pertumbuhan selama 20 jam pada 30°C dalam media MRS. Selain itu, keputusan menunjukkan bahawa TSB boleh menjadi media alternatif yang sesuai. Kuantifikasi aktiviti bakteriosin telah ditentukan dengan menggunakan microtiter plat assay. *L. lactis* subsp. *lactis* S20 menunjukkan kebolehan untuk proses penapaian sukrosa dan xylose yang mana ianya adalah bukan kebiasaan untuk *L. lactis*. Bakteriosin S20 menunjukkan aktiviti antibakteria ke atas kebanyakan patogen makanan Gram-positif tetapi tidak terhadap bakteria Gram-negatif. Keputusan ujian kestabilan menunjukkan bahawa bakteriosin S20 adalah tahan trypsin, amylase dan lipase tetapi sensitif kepada proteinase K, α-chymotrypsin dan pepsin. Bakteriosin ini adalah stabil dalam pelbagai pH (pH 3-9) dan pada suhu sehingga 70°C selama 15 min. Aktiviti bakteriosin daripada bakteriosin S20 menurun ke 40 AU/ml selepas disteril pada 121°C selama 15 min. Keputusan daripada ujian kestabilan dan kajian molekular mencadangkan bahawa bakteriosin S20 adalah berkait rapat dengan nisin A. Pengekstrakan bakteriosin S20 telah dijalankan dengan menggunakan kaedah sel-penjerapan. Berdasarkan kajian penjerapan bakteriosin, pH 6 dan pH 7 adalah pH yang optimum bagi penjerapan bakteriosin S20. Sel-sel strain-sensitif menunjukkan penjerapan bakteriosin S20 yang lebih tinggi daripada strain-produser. Walau bagaimanapun, penjerapan pada strain-tahan dalam kajian ini mencadangkan bahawa bakteriosin S20 terlekat secara rawak pada membran sel. Penjerapan bakteriosin dalam media TSB adalah lebih tinggi daripada yang di dalam media MRS. Sebaliknya, hasil dari pemulihan (*recovery*) dan penceriaan (*purification*) berganda yang lebih tinggi dilihat dalam bakteriosin S20

yang diekstrak daripada media MRS. Bakteriosin S20 telah menunjukkan kawalan secara efektif terhadap sel-sel *Listeria* dalam sistem susu (susu UHT). Selepas sel-sel *Listeria* dikurangkan dengan kehadiran bakteriosin S20, kiraan sel *Listeria* kekal sepanjang tempoh penyimpanan 14 hari. Dapatan kajian ini juga menunjukkan bahawa kiraan sel *Listeria* dalam sampel susu mengandungi 320 AU/ml bakteriosin S20 mempunyai sedikit atau tidak ada perbezaan yang signifikan (p>0.05) dengan kiraan sel *Listeria* dalam sampel susu mengandungi 160 AU/ml. Di samping itu, bakteriosin S20 menunjukkan aktiviti antilisterial yang berkesan dalam susu skim yang mengandungi <12% (w/v) susu pejal-bukan-lemak (*milk solids-not-fat* (MSNF)). MSNF dicadangkan mempunyai interaksi dengan bakteriosin S20 serta menjejaskan aktiviti antimikrobial bakteriosin S20 dalam susu. Kesimpulannya, bakteriosin S20 yang berjaya dipencilkan daripada sauerkraut Cina mempunyai potensi untuk digunakan sebagai antilisterial dalam makanan.

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I certify that a Thesis Examination Committee has met on 19 February 2016 to conduct the final examination of Chin Yih Zhet on his thesis entitled "Characterization of Bacteriocin Produced by *Lactococcus lactis s*ubsp. *Lactis* S20 Isolated from Chinese Sauerkraut and its Antilisterial Activity in Milk" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ABC transporter	ATP-binding cassette transporter
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU	Bacteriocin unit
A_w	Water activity
BHI	Brain heart infusion
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
LAB	Lactic acid bacteria
MIC	Minimum inhibitory concentration
MRS	de Man, Rogosa and Sharpe medium
MSNF	Milk Solids-not-fat
PCR	Polymerase chain reaction
PES	Polyethersulfone
rDNA	Ribosomal deoxyribonucleic acid
subsp.	Subspecies
Stx	Shiga-like toxin
TSB	Tryptic soy broth
UHT	Ultra-high temperature processing

CHAPTER 1

INTRODUCTION

Sulfites, benzoates, nitrites, and nitrates, it is not difficult to find these names in the ingredient column of the food products label. These chemical preservatives serve to slow down spoilage as well as improving food safety, by limiting the number of microorganisms within the foods. Although these chemical preservatives are verified by FDA as safe for the majority of the population, these chemical preservatives might cause negative side-effects as well (Dellavalle et al., 2014; Bryan et al., 2012; Vally and Misso, 2012; Zengin et al., 2011). Many food scientists are searching natural preservatives to cope with the heightened public awareness. Some of the newest antimicrobials have been found in microorganisms themselves as they form their own chemical defenses when competing for space and nutrients. Bacteriocin from Lactic Acid Bacteria (LAB) is one of the most promising natural preservatives discovered in the last few decades. This gene encoding antimicrobial peptides have attracted widespread attention among the scientific community in recent years for their potential applications in food preservation and improved safety and quality of food. The genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* have been associated with more than 300 different bacteriocins (Todorov et al., 2011). These bacteriocinogenic bacteria strains have been isolated from various environmental sources, and among them, fermented food being one of the most frequently studied. Although many bacteriocins produced by LAB are effective towards food borne pathogens and spoilage bacteria, to date only nisin is licensed for food use.

In Malaysia, Tianjin preserved cabbage (*tung tsai*), Chinese sauerkraut (*suan cai*), pickled cucumber, and pickled mustard stem (*zha cai*) are popular pickled vegetables among the Chinese community. It is very common for Chinese households make their own pickled vegetables, such as Chinese sauerkraut which is a pickled mustard green. In this case, mustard green is preserved in a brine medium and allowed to ferment under anaerobic conditions in a sealed clay jar at room temperature (25-28°C). The temperature, salty environment, and anaerobic condition favor the growth and predominance of LAB among the microbiota. The acid produced by the LAB lowers the pH of the fermentation process, which in turn inhibits the growth of certain food spoilage and pathogenic bacteria. An acidic environment commonly plays a critical role in preserving food from spoilage, but it has also been shown that certain microbial by-products, such as bacteriocins, inhibit some food spoilage and pathogenic bacteria (Riley and Gillor, 2007).

Cells of the bacteriocin producer and sensitive strains have been shown to possess the ability to adsorb and release bacteriocin at certain pH (Todorov et al., 2011; Todorov and Dicks, 2009; Burianek and Yousef, 2000; Yang et al., 1992). This has provided a simple, low cost and rapid tool to extract a large amount of bacteriocin from the production medium. The surface charge of the cell membrane is known to play a vital role in the adherence of the bacteriocin to the cell membrane. The adherence of the bacteriocin to the specific target receptor of the cell membrane is critical as it is this adherence which may have a lethal effect on the sensitive strain. However, not all bacteriocin-bound receptors cause a lethal effect (Todorov et al., 2007); for example, the adherence of bacteriocins to the producer and Gram-positive-resistant strains is non-lethal because the latter are protected by an immunity system orchestrated by the immunity gene clusters (Bastos et al., 2015). In addition, Gram-negative resistant strains are often protected by its outer membrane which plays an important role in preventing bacteriocins from gaining access to the cytoplasmic membrane (Gong et al., 2010). Due to the ability of bacteriocin to inhibit the food pathogen and spoilage, it has been applied commercially into food system.

Food is known as a complex system with various nutrients for human need. Many food processing methods, such as canning and pasteurization have been carried out to improve food safety as well as to preserve the food shelf life. Although pasteurization kills pathogens and the consumption of non-pasteurized dairy products is uncommon, dairy-associated disease outbreaks continue to occur (Langer et al., 2012). Many dairy products are marketed as pasteurized but the source of contamination is frequently traced to improper pasteurization, storage, or handling of the products. Bacteriocins could provide an extra protection against the pathogens. However, the effectiveness of bacteriocins are always in doubt when they come to the application in food. Studies have revealed that milk fat can influence the efficacy of bacteriocins. Jung et al. (1992) reported that nisin activity against *Listeria monocytogenes* fell by more than 88% in milk containing 12.9% milk fat. Bhatti et al. (2004) reported that the presence of phospholipids in the milk fat tends to bind a large portion of nisin, resulting in reduced nisin availability for antimicrobial activity. Zapico et al. (1999) also reported a reduced effect of nisin in the presence of milk fat. Nevertheless, the influence of milk solids-not-fat (MSNF) on the efficacy of bacteriocin has not been extensively investigated even though the influence of various MSNF levels is particularly important for the application of bacteriocins in dairy foods, such as ice cream, cheese, yogurt, and kefir.

Dozens of bacteriocins produced by genera of LAB have been reported and well documented. However, most bacteriocin-producing LAB were isolated from limited food sources and solely animal-associated food, for example, cheeses, meat, and milk. There are lack of article reported the isolation of bacteriocin-producing LAB strain from traditional pickled vegetable which is a good source of many beneficial bacteria. Research on the bacteriocins of new strains from different sources is therefore important in the search for bacteriocins with a better potential for applications in the food industry.

The objectives of this study are as follows:

1. To isolate bacteriocin-producing LAB from traditional pickled vegetables: Tianjin preserved cabbage (*tung tsai*), Chinese sauerkraut (*suan cai*), pickled cucumber, and pickled mustard stem (*zha cai*)
2. To determine the characteristics of the selected bacteriocin-producing LAB and its bacteriocin
3. To evaluate the antilisterial activity of the selected bacteriocin in milk

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