MODIFICATION OF SIGNAL PEPTIDE FOR ENHANCED SECRETION IN *Lactococcus lactis* FOR ORAL VACCINE DELIVERY

By

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Chairman : Prof. Datuk Wira Raha binti Haji Abdul Rahim, PhD
Faculty : Biotechnology and Biomolecular Sciences

Recombinant heterologous protein secretion is essential in biomanufacturing. Successful protein secretion generally depends on the host strain, expression and secretion machinery, and the target protein. *Lactococcus lactis*, which has a long history of safe use in food production, has been the workhorse for the secretion of various recombinant heterologous proteins, owing to its monolayer cell wall and presence of only one extracellular housekeeping protease with very few native extracellular proteins for simple and cost-efficient product recovery. Nonetheless, protein secretion in prokaryotes such as *L. lactis* is often plagued by several limitations such as incomplete translocation, protein misfolding, and degradation, leading to low secretion efficiency. In this study, the main aim is to enhance the low secretion efficiency (SE) in *L. lactis* by optimization of the secretion system utilizing a novel heterologous signal peptide (SP) SPK1 of *Pediococcus pentosaceus*. SPK1, which was previously shown to aid comparably, if not better secretion of heterologous proteins than the most widely used lactococcal signal peptide, USP45 was subjected to site- directed mutagenesis (SDM) of its amino acid sequence targeting the tripartite N-, H-, and C- terminal domain, respectively. The effect of SDM on SE was primarily tested on a model protein, *Staphylococcus aureus* nuclease (NUC). *In silico* analysis performed on the SPK1 yielded eight putative SPK1 variants; the cassettes of different SPs fused to NUC were cloned in nisin-induced pNZ8048 expression plasmid and introduced into *L. lactis* NZ9000 host. Analysis of secretion efficiency via Fluorescence Resonance Energy Transfer (FRET) activity assay revealed four of eight SPK1 variants carrying C-domain mutations had successfully enhanced SE compared to both control SPs, SPK1, and native lactococcal USP45. Additionally, one SPK1 variant (SPKM19) showed improved SE by approximately 88% or 1.3-fold than the wild-type SPK1. A subsequent fusion of the SPKM19 with a synthetic propeptide, LEISSTCDA, had further increased the SE. Subsequently, the efficiencies of the SPKM19-LEISS and SPK1-LEISS were further tested on two different therapeutic peptides; a modified 68-V (a derivative of G12V mutant KRAS) fused to carriermolecule diphtheria toxoid (68-V-DT) and a wild-type KRAS (wtKRAS). The SE of the secreted KRAS peptides was determined *in vitro* and *in vivo* via oral immunization with

mucoadhesive and enteric-coated *L. lactis*-secreting KRAS in BALB/c mice. Post-immunization assessments on the recombinant *L. lactis* secreting-KRAS aided by the optimized SPKM19-LEISS revealed a significant elevation in KRAS-specific intestinal IgA titer, indicative of positive induction of humoral immunity. Additionally, despite the lower immune responses observed for SPKM19-treated groups compared to the original SPK1-treated groups, which was consistent with the *in vitro* findings, secretion of the fusion peptide aided by both SPs to the targeted mucosal site was successfully shown. Altogether, this study demonstrated the development of an enhanced secretory system in *L. lactis* NZ9000 through an optimized signal peptide SPK1 (SPKM19) and LEISSTCDA, for heterologous protein production and oral vaccine delivery applications. Apart from that, this study also discussed the potential bottlenecks in developing the lactococcal GRAS (Generally-Regarded as Safe) as a secretory host for oral vaccine delivery targeting the mucosal environment.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MODIFIKASI ISYARAT PEPTID BAGI MENINGKATKAN REMBESAN DI DALAM *Lactococcus lactis* UNTUK TUJUAN PENGHANTARAN VAKSIN ORAL

Oleh

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Penghasilan rekombinan protein heterologus yang dirembeskan keluar adalah penting dalam sektor bioteknologi. Kejayaan rembesan protein heterologus secara amnya bergantung kepada faktor-faktor seperti perumah, jentera ekspresi dan rembesan, dan sasaran protein. *Lactococcus lactis*, yang mempunyai sejarah panjang penggunaaan selamat dalam pengeluaran makanan, telah digunakan sebagai ‒jentera hidup‖ bagi merembeskan pelbagai rekombinan protein heterologus. *L. lactis* memiliki kelebihan iaitu ia mempunyai satu lapisan dinding sel dan satu protease pengemasan luar selain penghasilan hanya beberapa protein luar menyebabkan proses ekstraksi yang mudah dan menjimatkan kos. Walau bagaimanapun, rembesan protein di dalam prokariotseperti *L. lactis* sering terbatas oleh faktor-faktor seperti translokasi yang tidak lengkap, penggulungan protein yang tidak betul, dan degaradasi protein, yang membawa kepada rembesan yang tidak cekap. Dalam kajian ini, tujuan utama adalah untuk meningkatkan kecekapan rembesan (SE) yang rendah dalam *L. lactis* dengan mengoptimumkan sistem rembesan menggunakan peptid isyarat (SP) heterologus baru, SPK1, daripada *Pediococccus pentosaceus*. SPK1, yang sebelum ini terbukti menunjukkan kemampuan yang setanding atau lebih baik berbanding peptid isyarat yang paling banyak digunakan dalam *lactococcal*, USP45, bagi perembesan protein heterologus, telah disasarkan sebagai Mutagenesis-Langsung Tapak ‒Site-directed Mutagenesis‖ (SDM) ke atas jujukan asid amino melalui tiga bahagian, domain-N, domain-H, dan, domain-C. Kesan SDM kepada kecekapan rembesan (SE) telah diuji pada model protein, *Staphylococcus aureus* nuclease (NUC). Analisis *in silico* yang dilakukan ke atas SPK1 telah menghasilkan lapan varian SPK1. Kaset-kaset yang berbeza SP dicantum kepada NUC gene telah diklon ke dalam plasmid ekspresi induksi-nisin pNZ8048 dan ditransformasikan ke dalam hos *L. lactis* NZ9000. Analisis kecekapan rembesan melaluiujian aktiviti ‒Fluorescence Resonance Energy Transfer (FRET)‖ menunjukkan empat daripada lapan varian SPK1 yang memiliki mutasi domain-C telah berjaya meningkatkan SE berbanding kedua-dua SP kawalan SPK1 dan USP45. Selain itu, satu varian SPK1 (SPKM19), telah menunjukkan peningkatan SE sekitar 88% atau 1.3 kali

ganda daripada SPK1 liar. Gabungan SPKM19 berikutnya dengan propeptid sintetik, LEISSTCDA, telah meningkatkan lagi SE. Selepas itu, kecekapan SPKM19- LEISS dan SPK1-LEISS diuji dengan lebih lanjut menggunakan dua peptid terapeutik berbeza; 68-V (derivatif G12V KRAS) yang diubahsuai dengan gabungan molekul pembawa Diptheria toxoid (DT), serta KRAS liar (wt-KRAS). Kecekapan rembesan dari peptid KRAS yang dirembeskan diuji secara *in vitro* dan *in vivo* melalui imunisasi oral dengan rekombinan *L. lactis*-merembeskan KRAS yang disaluti pelekat mukosa dan gabungan bersalut enterik ke dalam tikus BALB/c. Penilaian pasca imunisasi ke atas rekombinan *L. lactis*-merembaskan KRAS yang dibantu oleh SPKM19-LEISS menujukkan peningkatan yang signifikan pada titer IgA usus spesifik-KRAS, yang menunjukkan induksi positif terhadap imuniti ‒humoral‖. Selain itu, walaupun tindakbalas imun yang rendah diperhatikan untuk kumpulan yang dirawat SPKM19 berbanding dengan kumpulan yang dirawat SPK1 yang asal, yang selaras dengan penemuan *in vitro*, rembesan gabungan peptid yang dibantu oleh kedua-dua peptid isyarat (SP) ke laman mukosa yang disasarkan berjaya ditunjukkan. Secara keseluruhan, kajian ini menunjukkan penghasilan sistem rembesan yang dipertingkatkan dalam *L. lactis* NZ9000 melalui peptid isyarat yang dioptimumkan, SPK1 (SPKM19), dan LEISSTCDA, bagi tujuan pengeluaran protein heterologus dan aplikasi penghantaran vaksin oral. Selain itu, kajian ini juga membincangkan potensi GRAS (Secara Umum-dianggap sebagai Selamat) *lactococcal* sebagai hos perembesan bagi tujuan penghantaran vaksin oral yang menyasarkan persekitaran mukosa.

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~	approximately
°C	degree Celsius
µF	microliter
6x-His	six-histidine
aa	amino acid
Abs	Absorbance
Ala	Alanine
Amp	ampicillin
BHI	Brain heart infusion
BHQ2	Black Hole Quencher 2
bp	base pairs
BSA	Bovine Serum Albumin
BSA	Bovine serum albumin
CaCl2	calcium chloride
CFU	Colony-forming unit
cm	centimeter
Cm	Chloramphenicol
CRC	colorectal cancer
CTL	Cytotoxic T cell
Cy3	Cy3 fluorophore
Da	Dalton
DAB	3,3'-Diaminobenzidine
dH20	distilled water
DNA	deoxyribonucleotide acid
EDTA	ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
FRET	Fluorescence Resonance Energy Transfer
GALT	Gut-associated lymphoid tissue
GFP	green fluorescent protein
GIT	gastrointestinal tract
Gly	Glycine
GM17	M17 supplemented with 0.5% glucose
GRAS	Generally Regarded as Safe
GRAVY	Grand average of hydropathicity
h	hour
H_2SO_4	sulfuric acid
HRP	Horse Radish Peroxidase
HRP	Horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Ile	Isoleucine
Kan	kanamycin
kb	kilo base pairs
kDa	kilo Dalton
KRAS	Kirsten ras
kV	kilovolt
l	liter
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
LEISS	LEISSTCDA

Lys	Lysine
M	Molar
mA	milliampere
mCRC	metastatic colorectal cancer
MCS	multiple cloning site
mg	milligram
MgCl2	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MP	mature protein
mut	mutant
NCBI	National Center for Biotechnology Information
ng	nanogram
NICE	Nisin Controlled Gene Expression
nis	nisin
nm	nanometer
nM	nanomolar
NNs	neural networks
NSP	native signal peptide
NSP	native signal peptide
NTA	nitriloacetic acid
NUC	nuclease
OD	Optical Density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween® 20

PCR	Polymerase Chain Reaction
pI	isoelectric point
P_{nisA}	nisA promoter
pre	precursor protein
Pro	Proline
psi	pounds per square inch
RBS	ribosomal binding site
RE	Restriction enzymes
RFU	relative fluorescence units
RNA	ribonucleotide acid
rpm	revolutions per minute
RT	room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
SE	secretion efficiency
sec	seconds
Sec-dependent	Secretory-dependent
Ser	Serine
SIgA	Secretory IgA
SP	signal peptide
SPase	signal peptidase
Ta	annealing temperature
TAA	tumor-associated antigen
TBD	Toluidine blue DNA agar
TCA	Trichloroacetic acid
Th	T helper cell
Thr	Threonine

Tregs	Regulatory T cells
Tris	Tris(hydroxymethyl)aminomethane
TSA	tumor-specific antigen
UV	ultraviolet
V	volt
v/v	volume per volume
Val	Valine
w/v	weight per volume
wtKRAS	wild-type KRAS
x g	times gravity
µg	microgram
µl	microliter

CHAPTER 1

INTRODUCTION

Research on recombinant secretory protein has intensified in the last decade to meet the ever-growing need for high yield, quality, and active recombinant proteins for newly isolated enzymes and various other economically and pharmaceutically valuable proteins. The secretory expression system used for the manufacturing of these heterologous proteins must meet specific criteria such as consistent product quality and cost-effectiveness. Secretion, which direct translocation of recombinant protein across plasma membrane directly into culture media, offers fast and straightforward purification and permits direct excess to the targeted environment (Pohl and Harwood, 2010), thus becoming a better means for vaccine delivery and large-scale industrial applications (Song et al., 2017).

Various expression systems have been employed in pharmaceutical industries, including prokaryotes, yeasts, filamentous fungi, insect, and mammalian cell culture. Meanwhile, bacterial secretory expression systems are prevalent for heterologous protein production and vaccine delivery due to their ability to grow rapidly and at high density on inexpensive substrates. Besides, their genetics and biochemistry are well understood and are commercially available. *Lactococcus lactis* is one of the most favored and important Gram-positive prokaryotic cell factories for heterologous protein production besides hosts such as *Bacillus subtilis*, or Gram-negative *Escherichia coli* counterparts, owing to its monolayer cell well, absence of inclusion bodies and simple proteolytic system comprising of only one extracellular housekeeping protease, HtrA and one extracellular native protein, USP45 (Le Loir et al., 2005). Not only that, this lactic acid bacteria, traditionally used for centuries in the food production industry, has also advanced its uses as a vaccine delivery carrier owing to its GRAS status, probiotics and immunomodulating properties (Wang et al., 2016). Numerous advancements for expression developed in *L. lactis* have allowed for high production ofexpressed proteins intracellularly. Nevertheless, highly expressed intracellular proteins often impose certain limitations such as protein aggregation and misfolding, and expensive downstream purification of products (Baradaran et al., 2013). Therefore, many studies have now shifted to protein secretion as a means to overcome those bottlenecks.

As much as secretion is the preferred strategy for high yield of heterologous proteins and for vaccine delivery in *L. lactis*, thereby enabling high yield of protein products (Fernandez et al., 2009), difficulties in obtaining optimal secretion efficiency remains a major challenge for secretion in many prokaryotic hosts including *L. lactis* (Zhang, Zhong, and Liang 2010). The secretion efficiency (SE), which is defined as the proportion of secreted proteins as opposed to the total protein produced, is most often reported to be insufficiently low in the lactococcal host. Several factors such as inefficient translocation by the secretory machinery, improper protein folding, and protein degradation by the host proteases could be causing the low secretion efficiency of secreted proteins. Those factors are highly influenced by the nature of signal peptide, mature protein, and the host strain, respectively (Le Loir et al., 2005; Westers, Westers, and Quax, 2004).

Signal peptide (SPs), which is an N-terminal signaling sequence located upstream of a mature protein (MP) region, often shares a conserved tripartite structure; the positively charged N-terminal, the hydrophobic core of H-domain, and the non-polar C-terminal containing cleavage site for signal peptidases. Each of the domain regions is known to play a specific role in the signal peptide export function. Additionally, each signal peptide reportedly has varying efficiency across different proteins used (Freudl, 2018). Various random or site-directed mutagenesis targeting the tripartite structure have been conducted to improve the performance of the SP such as observed for the most successful lactococcal signal peptide, USP45, which optimization of its inherent limitations had improved secretion yield by 51% compared to that of wild type USP45 (Ng and Sarkar, 2013), yet the SE of heterologous protein was still not optimally achieved in *L. lactis*. On the other hand, several new homologous SPs in *L. lactis* such as SP310 (Ravn et al., 2000), SPExp4 (Morello et al., 2008), SPAL9 (Ravn et al., 2003) had been developed, yet none of them were able to mount higher SE than the lactococcal USP45, even after the engineering of the signal peptides such as observed for SP310mut2 (Ravn et al., 2003). Subsequently, the use of heterologous SP such as the SLPA of *Lactobacillus brevis* was reportedly able to improve SE in *L. lactis*. Yet, the total protein remained lower when compared to the lactococcal USP45 (Zhang et al., 2010). Therefore, developments of new SPs for applications in *L. lactis* are still impeding.

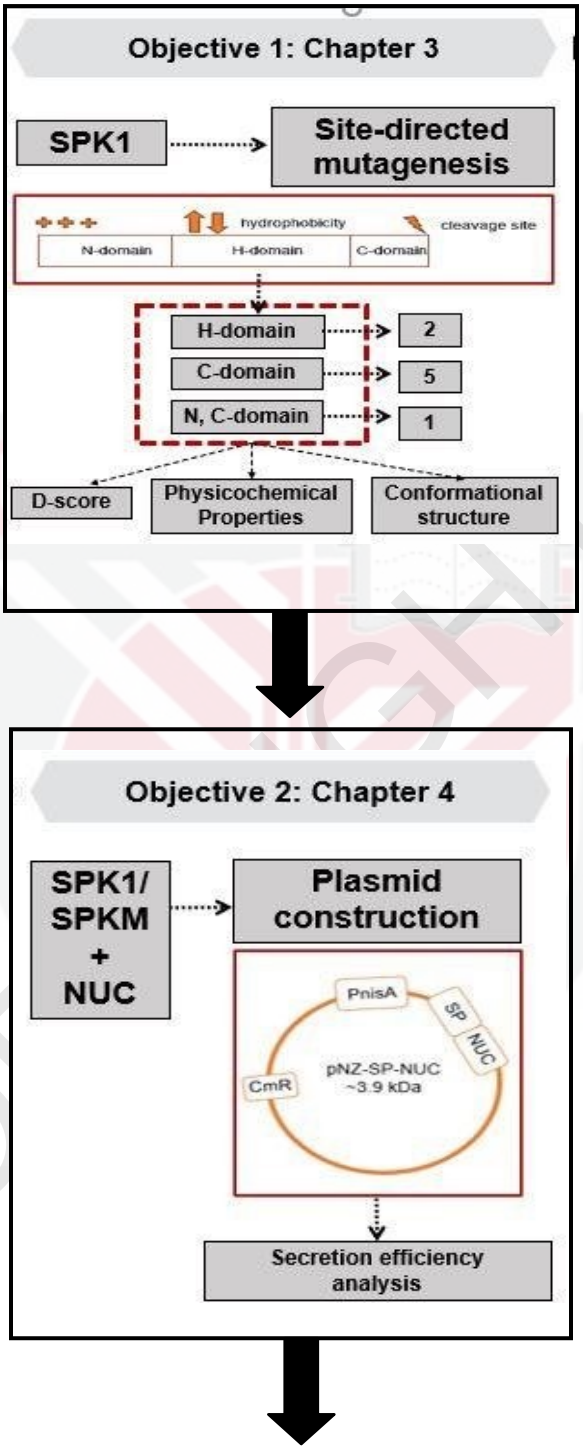
In view of this, recently, Baradaran et al. (2013) had isolated a novel heterologous signal peptide SPK1 of *Pediococcus pentosaceus* with the ability to produce comparably if not better secretion efficiency than that of the native and well- established lactococcal USP45. *In silico* and physicochemical analyses studied by Baradaran et al. (2013), showed there is a potential to further enhance this signal peptide capacity for higher secretion efficiency of heterologous proteins in *L. lactis*. The SPK1 had been previously shown to mediate comparable secretion yield for a reporter GFP protein (Baradaran et al., 2013), and higher SE for several industrially- important heterologous proteins such as β-cyclodextrin glucanotransferase (β-CGTase) (Subramaniam et al., 2013) and xylanase (Roslan et al., 2020) enzymes. The SPK1 is thus, a promising SP to be further studied for enhanced secretion in *L. lactis.*

Therefore, in this study, the low secretion efficiency in *L. lactis* was circumvented by utilizing the heterologous signal peptide, SPK1 of *P. pentosaceus*. The amino acid sequence of SPK1 was optimized by subjecting site-directed mutagenesis (SDM) on the tripartite N-, H-, and C- domains, respectively, and by fusion with an anionic linker, LEISSTCDA, to further enhance the SP efficiency. LEISSTCDA is an anionic short propeptide that was previously demonstrated to be able to increase further secretion efficiency of secreted proteins in *L. lactis* (Ribeiro et al., 2002). The effect of SDM was primarily tested on the secretion of a model protein, *Staphylococcal* nuclease (NUC), which rapid assay for screening of the enzymatic activity via rapid and sensitive petri dish plate assay is widely available. Subsequently, to further develop the *L. lactis* as a potential mucosal vaccine delivery host, the efficiency of the best selected mutant SPK1 was further tested on two novel therapeutic KRAS peptides, a 68-V mimotope fused with Diptheria toxoid (68-V-DT) and its control, wild type KRAS (wtKRAS). KRAS is an oncogene that is most frequently mutated in colorectal, pancreatic, and non-small-cell lung cancers (NSCLCs) (Fernández-Medarde & Santos, 2011).

Meanwhile, the 68-V mimotope is a modified mutant KRAS G12V with an additional point mutation at V7D position, which had been previously demonstrated to confer immunomodulant property (Ng et al., 2018). The fusion of the mimotope with a carrier molecule, diphtheria toxoid (DT) in this study would add to the chemical stability and adjuvancity. Thus, the 68-V-DT served as a potential cancer vaccine candidate. Through selection of one of the best SPK1 derivatives fused with an anionic linker, LEISSTCDA, the peptides were expressed and secreted in *L. lactis* before orallydelivered into BALB/c mice for assessment of secretion and immunogenic responses *in vivo*. Additionally, a comparative analysis was conducted *in silico*, to understand further the effects of different SPs with different fusion mature proteins secretion in *L. lactis*.

In summary, the main objective of this study was to enhance secretion efficiency in *L. lactis* by modification of signal peptide SPK1 for heterologous protein production and vaccine delivery. Meanwhile, the specific objectives of this study were:

1) to design site-directed mutagenesis candidates on SPK1 and determine the characteristics of SPK1 variants,
2) to construct and determine the secretion efficiencies of *L. lactis* recombinants harboring different SPs fused with NUC,
3) to construct and determine the secretion performances of two heterologous KRAS epitopes fused with the best SPK1 variant and LEISSTCDA in *L. lactis*,
4) to determine the immunogenicity of orally delivered recombinants *L. lactis* secreting the KRAS epitopes in BALB/c mice.



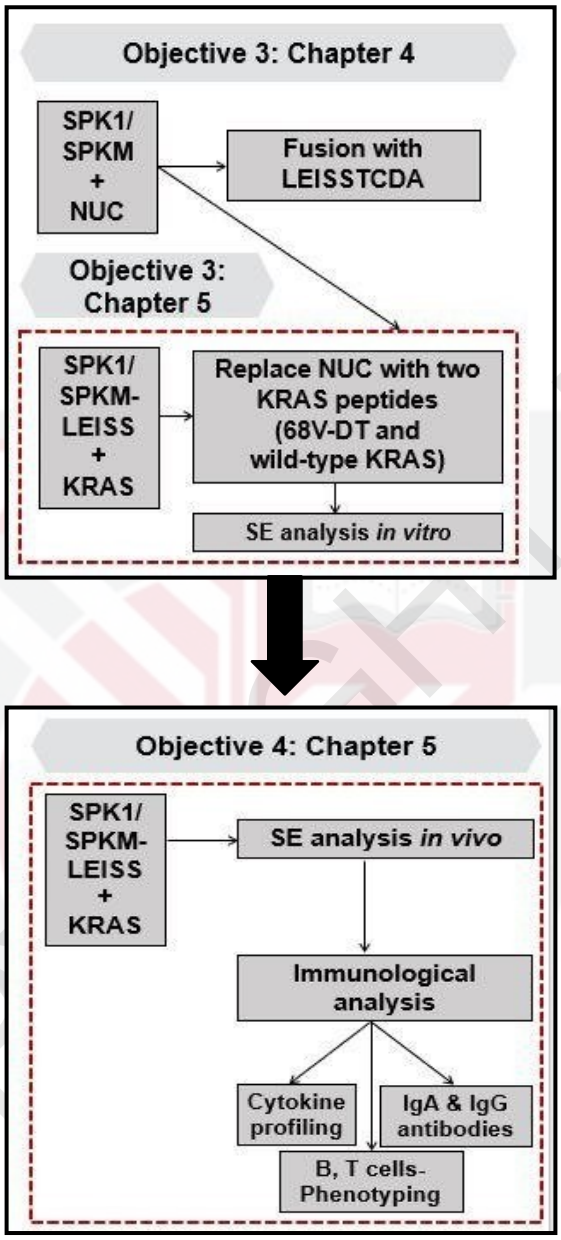


Figure 1.1 : Flow chart of the overall study. Site-directed mutagenesis of SPK1 via N, H, and C-domain regions produced different SPK1 variants whereby each were characterized by *in silico* analysis based on several parameters (Objective 1). The putative SPK1 mutants were fused with a model protein, NUC, and the constructed recombinants *L. lactis* were subjected to SE analysis (Objective 2). One of the best mutants was selected and it was fused with LEISSTCDA to further enhanced SE prior to replacement of the model protein with new proteins, two KRAS antigens, for oral vaccine delivery. SE of the different *L. lactis* secreting KRAS recombinants were analyzed *in vitro* (Objective 3) and *in vivo* using Balb/C mice model (Objective 4).

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