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Expression of an antimalarial peptide by plasmid transfected Yogurt bacteria I: feasibility, experimental design and measurement

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Abstract

The feasibility of making 'antimalarial yogurt' was explored. A plasmid containing the gene for the antimalarial peptide, NK2, linked to a nonapeptide expressing linker and a gene expressing a signal peptide USP45 to facilitate the export of NK2 out of the bacterial cell; an antibiotic resistance gene as well as a gene expressing the protein UL16 to render the bacterial cell resistant to the expressed NK2 was designed to be cloned into *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*; the two bacterial species present in the yogurt of choice. An inexpensive electroporation apparatus was identified for transformation of the yogurt bacteria with the plasmid. Yield calculations showed that an adult would need to ingest two 5.3 ounce servings of recombinant yogurt per day to achieve a therapeutic concentration of NK2 in blood. Expression of NK2 was demonstrated by performing preliminary hemolysis experiments using sheep blood with and without incorporation of yogurt. A literature search indicated that *Plasmodium bergheii,* a species that is non-infective in humans, could be cultured *in vitro* using sheep blood thus eliminating the need to procure and house live rodents. The school chemistry laboratory was classified as being biosafety level 1, thereby allowing material transfer agreements with vendors shipping the plasmodium parasite, plasmids and blood.

Introduction

An antimicrobial peptide consisting of the α-helical cationic core region of mammalian NK-Lysin, designated as NK-2, has been reported to be effective at killing intraerythrocytic *Plasmodium falciparum* at μM concentrations (1, 2). NK-2 is active against blood-stage Plasmodia at physiological salt concentrations in the presence of serum components. Its hypothesized mechanism is by binding to the (increased) concentration of phosphatidyl serine (PS) at the outer bilayer membrane of the infected red blood cell (RBC). Since the PS of non-infected erythrocytes is found predominantly in the inner bilayer membrane (3), NK-2 has no

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hemolytic effect on non-infected RBC. The idea of incorporating the complementary DNA (cDNA) of this peptide into yogurt bacteria has been previously elucidated in the literature (4). Among the many advantages of such an approach to combat malaria is the ease of adoption by economically, politically and/or geographically disadvantaged populations. In addition, this 'open source' medicine can be used for as long as the plasmid is capable of replication.

Before undertaking a project of this scale, it is worthwhile designing the experiment(s) such that they are reproducibly quantifiable, sound and robust within the parameters of funding, resources and equipment available. Some of the questions that need answers are:

1. Can the cDNA of the NK-2 peptide be incorporated into a suitable plasmid expression vector?
2. Can Yogurt bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*) be transformed by this plasmid to express NK-2 *in vitro* in sufficient amount to kill *Plasmodium berghei* parasites? What relative amounts of yogurt and blood must be contacted to achieve an effective concentration for 50% inhibition (ED_{50}) for the expressed NK-2 peptide? Do these relative amounts translate into a reasonable amount of yogurt ingestion per day?
3. Can the recombinant NK-2 be transported outside the bacterium and secreted into the intestine using a suitable signaling sequence, without forming inclusion bodies and without over-taxing the metabolic rate of the bacterium?
4. Is funding sufficient to have an external laboratory prepare a suitable plasmid vector, to purchase *Plasmodium berghei* and have equipment and materials for amplification/ passage of the parasite until the experiment is complete? Can the parasite be cultured/amplified *in vitro* thereby eliminating the need to acquire and house live animals?
5. Are an autoclave, centrifuge, deep freezer, refrigerator, incubator, electroporator, spectrophotometer, agar medium and agar plates available?
6. Can the hemolysis of sheep blood be used as a surrogate marker to measure the effectiveness of the NK-2 peptide in killing erythrocytes infected with *Plasmodium berghei*?
7. Can the yogurt ingredients (solids, proteins) and/or yogurt bacteria by themselves cause or inhibit hemolysis of sheep RBC thereby causing interference with analysis and confounding results. If yes, can these confounding factors be removed or controlled?
8. Since NK-2 has antibacterial properties, what is the probability that it will kill transformed *Lactobacillus delbrueckii subsp. bulgaricus* and/or *Streptococcus thermophilus*, so that it terminates its own expression? Are there way to circumvent this conundrum?

Before ordering expensive materials and performing time-sensitive experiments, it was deemed prudent to

determine whether hemolysis could be measured using a visible spectrophotometer, if yogurt solids or bacteria caused or inhibited hemolysis, if the school chemistry laboratory could be certified as being a Biosafety level 1 laboratory to enable material transfer agreements and shipping with vendors of plasmids, *Plasmodium berghei* and living tissue (blood), whether inexpensive electroporation apparatus could be constructed to transform yogurt bacteria and if the parasite could be cultured *in vitro* to eliminate the need to request regulatory approval for, acquire and house live rodents. This paper, representing the first of three anticipated manuscripts, details these considerations, analyses and calculations along with proof-of-principle measurements.

Materials:

Defibrinated sheep blood was obtained from Ward Science, Rochester, NY and stored at < 8^{o}C in a 3.8 ft^3 refrigerator. Pyrex® Culture tubes, 2 cm ID and a length of 1 cm were used to contact the blood with the various media studied. Weights greater than 1 g were weighed on a scale, model OB2141, d=0.01g, Flinn Scientific, Batavia, IL. Oikos® Greek nonfat yogurt certified by the National Yogurt Association, Live and active cultures seal (> 10^8 CFU per gram and fermented with both *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) (5) was purchased from a local Walmart store and kept refrigerated until use. Samples were centrifuged in a microcentrifuge (REVSCI Revolutionary Science, MN, Rev Spin 200T) in microcentrifuge tubes (Thumbs-up Microtubes, Diversified Biotech (Catalog #: THUM-200). A visible spectrophotometer, catalog No. AP7026, Flinn Scientific, was used to measure the absorbance of hemolyzed blood and/or clear supernatant in test tubes with an internal diameter of 1 cm and a length of 10 cm.

Sodium Chloride, lab grade, was obtained from Ward Scientific, lot#: 2013080824. Distilled water was prepared using a pressure cooker (Prestige Deluxe Alpha) placed on a Corning PC-400D hot plate set at the maximum heat setting, the hot water was fed through a Tygon® tube and condensed using a Climate keeper window fan into a 2 L plastic jug. A balance from OHAUS Pioneer™, d= 0.001g was used to weigh materials weighing < 1 gram. A Labquest Vernier Conductivity Probe Con-BTA, Vernier software and technology, Beaverton, OR, calibrated with a sodium chloride solution conductivity standard (500 µS cm^{-1}) was used to measure the conductivity of distilled water. A Labquest Vernier pH probe calibrated with pH 7.0 and 4.0 buffers was used to measure the pH of distilled water, Gram's Iodine Solution (Catalog #82-1051) and Crystal Ammonium Oxalate (Catalog #: 82-1051.1) were obtained from Carolina biological supply, Burlington, NC, as were the micropipettes (100-1000 µL) used for sampling.

Methods:

Identification of the cDNA sequence for NK-2

NK-2 has the amino acid sequence KILRGVCKKIMRTFLRRISKDILTGKK. Using the Basic Local Alignment Search Tool (BLAST™) database from the US National Library of Medicine (USNLM), returned 69 hits on 68 subject sequences. The top scoring sequence with a 100% identity was that for *S. scrofa* mRNA for NK-lysin (GenBank accession No. X85431.1). The base sequence for the core functional region of NK-Lysin began at nucleotide 468 and ended at nucleotide 554. The peptide itself has 185 amino acid residues and has a DNA sequence of 780 nucleotides (6). The cDNA sequence is:

aagatactgagaggtgtgtgcaagaagatcatgaggacctttctccgtcgcatctccaaggacatcctgactgggaagaaa

Using reverse translate (7), a cDNA sequence was generated representing the most likely non-degenerate coding sequence. The default codon usage table itself was generated using all the *Escherichia coli* coding sequences in GenBank. The sequence is:

aaaattctgcgcggcgtgtgcaaaaaaattatgcgcacctttctgcgccgcattagcaaagatattctgaccggcaaaaaa

The sequence from *S. scrofa* was codon optimized for *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ATCC 11842/DSM 20081 using the JCat codon optimization tool (8). The choice of the codon sequence for the target gene significantly influences the expression of the protein (9). The final cDNA sequence to be inserted into the plasmid expression vector is:

aagatcctgcgcggcgtttgcaagaagatcatgcgcactttcctgcgccgcatctcaaaggacatcctgactggcaagaag

Identification of a suitable secretion signaling sequence for NK-2

Recombinant heterologous proteins produced in Gram positive bacteria need to only traverse one membrane (instead of two in Gram negative bacteria), to enter the extracellular environment. Therefore, the formation of inclusion bodies is not usually as pronounced in Gram positive bacteria if the protein is tagged with a suitable secretory signal peptide. Several candidates can serve as such signaling peptides for Lactic Acid Bacteria (LAB). For example, a signal sequence from *Lactobacillus delbrueckii* subsp. *bulgaricus*; Part BBa_K128004 (10) was isolated from the 5' end of the prtB gene which codes for a lactose digesting protein that is bound to the surface (11). Another signal peptide, USP45, Part BBa_K1830002 (12), is a secreted extracellular protein of *Lactococcus lactis* that allows for extracellular secretion of any protein (13). A secretion signal peptide originated from the *Bacillus subtilis* extracellular protease AprE, Part BBa_K1674000 (14) is available commercially as part of a *Bacillus subtilis* secretory protein expression system (15). Secretion efficiency in *Lactococcus lactis* was increased when a synthetic nonapeptide (LEISSTCDA) was fused between the USP45 signaling sequence and the recombinant protein (16, 17), due to negatively charged residues from the synthetic peptide attached to the N-terminal end of the recombinant protein (18). For proteins containing two or more cysteine residues, fusion to an intrinsically disordered RO subdomain of the GLURP antigen (19) significantly increased expression from *Lactococcus lactis* (20).

Identification of a suitable malaria parasite and expression plasmid vector:

Plasmodium berghei was the parasite of choice for the project because it does not infect humans but can still infect other mammals, especially rodents. Therefore, the hemolysis of sheep blood can be followed using this organism (21) in a Biosafety level 1 certified laboratory. An in-house assessment was performed on the Chemistry laboratory (22) and it was determined that the laboratory met Biosafety Level 1 requirements. *P. berghei*, strain (ANKA) GFPCON 259cl2 is a genetically modified parasite derived from strain ANKA cl15cy1 following stable transfection with the pL0016 vector containing the GFP gene. It is available from the Biodefence and Emerging Infections Research Resources Repository (BEI resources) as a vial containing 0.5 mL of *P. berghei* -infected mouse blood in Glycerolyte 57 solution (1:2), catalog No. MRA-785. It can be ordered once a year gratis from BEI resources. Although the product information sheet states that the strain must be amplified in rodents, the literature indicates that *in vitro* passage and amplification without loss of infectivity is possible (23, 24). *Plasmodium berghei*, ATCC 30090 can also be purchased from American Type Culture Collection (ATCC), VA. The vector pLEM415 was chosen to transfect *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; the bacteria present in the yogurt because of its high transformation efficiency of the order of 10^4 transformants per µg of plasmid DNA for these bacterial species (25). In addition, the use of L-lactate dehydrogenase (ldhL) of *Lactobacillus sakei* (26) as a constitutive promotor in the pLEM415 backbone (designated as pLEM-ldhl), yielded high *in vivo* expression levels of exogenous proteins from *Lactobacilli delbrueckii* subsp. *bulgaricus* using this plasmid (27).

The sequence information of a pLEM415-ldhl-mRFP1 vector (plasmid no. 99845) was obtained from the nonnprofit plasmid repository , Addgene, Watertown, MA (28) and pasted in the free web based gensmart-design® application from Genscript, Piscataway, NJ. The plasmid contained an ampicillin resistance gene and its promoter in the backbone. The mRFP1 was deleted and the Jcat codon optimized cDNA sequences of the ldhl promoter, NK2, linker nonapeptide and the USP45 signal peptide were inserted into the pLEM415 backbone to make the plasmid vector product designated as pLEM415-ldhl-NK2-link-USP45.

Calculation of the amount of recombinant NK-2 expressed in yogurt bacteria:

Using the amount of recombinant protein expressed by a single bacterium:
The total protein content of a bacterial cell is estimated to be between 60 to 330 fg (29). Assuming a recombinant protein expression that is 5% of the total cellular protein (30), one bacterial cell will express ~ 10 fg of NK-2. Using a bacterial count of > 1 x 10^{10} CFU per gram of orally administered yogurt, 2.0 x 10^{-4} g of NK-2 is calculated to be expressed for 2 g of recombinant yogurt. Using a molar mass of 3671.4 g mol^{-1} for the NK-2 peptide, this translated into a concentration of 5.45 x 10^{-6} M (5.45 µM) in the 10 mL solution containing 2 g yogurt and 1 mL of sheep blood.

Using a cell culture density value from the literature:
Wang et.al. achieved expression levels of 17 to 34 mg of unlabeled protein from a 50 mL cell culture that reached an optical density at 600 nm (OD_{600}) of 10-20 using only an incubator shaker (31). Assuming that our study could achieve half the lower concentration of 8 mg from a 50 mL recombinant yogurt cell culture, 3.4×10^{-4} g of NK-2 was calculated to be expressed from 2 g of recombinant yogurt. This translated into a concentration of 9.26×10^{-6} M (9.26 µM) in the 10 mL solution containing 2 g yogurt and 1 mL of sheep blood.

Using a mammalian cell culture expression value:
Hippenmeyer et.al (32) could recover 1-20 µg of recombinant protein from a mammalian cell culture containing 1×10^{6} cells over a period of 24 hours. Using the average value, this translates into 1×10^{-4} g of NK-2 or 2.72×10^{-6} M (2.72 µM) in the 10 mL solution containing 2 g yogurt and 1 mL of sheep blood.

Using these three methods results in a similar order of magnitude of NK-2 concentrations. These micromolar range concentrations calculated above showed a strong hemolytic activity (50 to 75% compared to control) toward RBCs infected with *Plasmodium falciparum* but no activity toward non infected RBCs (33). These experiments were performed at a hematocrit of 2.5% at 37^{o}C for 2 hours. Since the lower level of sheep hematocrit is 27% (34), the experimental protocol will use [(the supernatant of the centrifuged) 2 parts by volume of yogurt diluted with 7.9 parts by volume of 1% sodium chloride solution] and 0.1 parts by volume of sheep blood (infected and non-infected) incubated for 2 hours at room temperature to determine efficacy of the recombinant yogurt (RY).

Identification of an inexpensive electroporator and electroporation method:

Electroporation is a convenient method to transfect bacteria with an expression plasmid. Electroporators are expensive pieces of equipment, typically costing thousands of dollars. A literature search identified an inexpensive electroporator made from a household piezoelectric gas lighter (35) with a transformation efficiency of 10^{5} transformants per µg of plasmid DNA carrying a green fluorescent protein (GFP) reporter gene into *Escherichia coli*. The efficiency was comparable to a commercial electroporator (BioRad MicroPulser®, BioRad Corp., Hercules, CA)

Feasibility calculations

Assuming an average adult blood volume of 5 L, at an effective concentration for 50% activity (EC_{50}) concentration of 6 µM, 30 µmole (0.11 g) of NK-2 was calculated to be present in the bloodstream for efficacy. As a first approximation, this translates to ingesting 324.7 g of yogurt, equivalent to about two 5.3 ounce cups per day, (assuming 100% absorption of the expressed NK-2 from the gastro-intestinal tract (GIT) into the bloodstream, see below for caveats and further refinement. In addition to the absorption of expressed NK-2, orally administered bacteria can themselves translocate out of the gastrointestinal tract and find their way into the

bloodstream. Commensal non-pathogenic transformed *Bifidobacterium breve* expressing *lux* orally administered to mice were detected in subcutaneous tumors at levels similar to intravenous administration (36). This presents another avenue for NK-2 to find its way into the bloodstream. The literature suggests that yogurt bacteria, especially *L bulgaricus* can survive for days in the human GIT as evidenced by their retrieval from feces of healthy individuals (37). Furthermore, orally administered recombinant *Lactococcus* and *Lactobacillus* species were effective in expressing IL-10 in the intestine to treat colon inflammation and inflammatory bowel syndrome (IBS) (38, 39), as oral vaccines to target specific peptides such as the HIV-1 epitope (40), *Bacillus anthracis* (41) and bovine β lactoglobulin (42), and against human papillomavirus type 16 (HPV-16) (43) induced tumors in mice. These studies demonstrated that yogurt bacteria can effectively express their genetically programmed protein cargo at mucosal surfaces *in vivo*. Furthermore, a plasmid rate loss of approximately half the administered amount was reported per day (44). Since the yogurt is replenished daily, this is not a problem. This also means that there is negligible chance of long-term harmful effects (if any) of the recombinant bacteria.

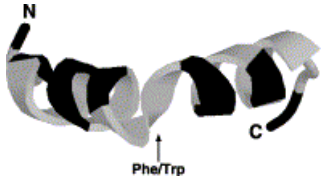
Using a Caco-2 cell monolayer permeability assay, Zheng et.al. (45) demonstrated that orally administered *Lactobacillus paracasei* recombinantly expressing the GLP-1 receptor agonist, exendin-4, showed a 34-fold increase in transport across the monolayer compared to free exendin-4. Exendin-4 is a 39 residue amino-acid peptide with a molecular weight (4186.7 Da) comparable with that of NK-2 (3671.4 Da). Therefore, conservatively assuming a 2% bioavailability (46) for the orally administered free NK-2 peptide, it is reasonable to assume a bioavailability of 64% for the recombinantly expressed NK-2 when administered orally since permeability values calculated using this *in vitro* model correlate well with human *in vivo* absorption data (47). This results in a downward revision of the initial first approximation of the blood concentration of NK-2 to 3.8 µM upon consumption of a 5.3 ounce cup of recombinant yogurt, twice a day. This concentration translates to a 50% hemolysis of infected erythrocytes at a parasitemia of 5% (48), enough for a significant antimalarial effect. However, since the estimated elimination half-life of the peptide in human reticulocytes is 1.5 hours and the peptide is classified as unstable per its calculated instability index of 45.43 (49), it may need to be tagged with protease resistant amino acid motifs to increase its elimination half-life.

The antibacterial activity of NK-2 against Lactobacillus delbrueckii subsp. *bulgaricus and Streptococcus thermophilus*

NK-lysin, the parent peptide of the core NK2 domain, showed high antibacterial activity against *Escherichia coli* and *Bacillus megaterium* and moderate activity against *Acinetobacter calcoaceticus* and *Streptococcus pyogenes* (50). It had no activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonellaspp*. while exhibiting some antifungal activity against *Candida albicans* (51). It was found that the broad-spectrum antibacterial activity of NK-2 and NK-2 analogs was the result of the possession

of a minimal positive net charge plus a highly amphipathic anchor point of two helical turns contributed by seven amino acid residues (52, 53), resulting in the peptide forming pores in lipid bilayers; a phenomenon termed molecular electroporation (54). It was also postulated that the binding of the wedge-shaped NK-2 to phosphatidylethanolamine (PE) increased the intrinsic negative curvature strain of a PE lamellar phase thereby disrupting the bacterial cell membrane structure (55).

Figure 1: The structure of NK-2, extracted from the NMR structure of NK-lysin at the protein data bank (1 NKL) (53). The cationic residues are shown in black.



NK-2 showed antibacterial activity against a wide spectrum of both gram positive (56) and gram-negative bacteria. It can thus be expected to cause lysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as well. However, recombinant NK-lysin has been successfully expressed in *Escherichia coli*; a bacterium that is susceptible to lysis by the peptide (57). Antibiotic peptides were produced in *Escherichia coli* transformed with a low to medium copy plasmid using promoters under both transcriptional and DNA control (58) such that their toxicity toward the expressing bacteria was minimized. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were reported to have low and intermediate sensitivity respectively to class IIa bacteriocins that target the proteins of the sugar transporter mannose phosphotransferase system of the bacterial membrane (59). A specific exopolysaccharide secreting strain of *Lactobacillus delbueckii* subsp. *bulgaricus* was reported to be resistant to class I bacteriocins such as nisin (60). Heterologous production of bacteriocins by lactic acid bacteria has been described with (61) or without simultaneous cloning of their analogous immunity genes (62). The human cytomegalovirus protein, UL16 was reported to increase resistance to natural killer cell cytotoxicity through resistance to cytolytic proteins including to NK-lysin (63). UL16 has the amino acid sequence:

MERRRGTVPLGWVFFVLCLSASSPCAVDLGSKSSNSTCRLNVTELASIHPGETWTLHGM
CISICYYENVTEDEIIGVAFTWQHNESVVDLWLYQNDTVIRNFSDITTNILQDGLKMRTV
PVTKLYTSRMVTNLTVGRYDCLRCENGTMKIIERLYVRLGSLYPRPPGSGLAKHPSVSA
DEELSATLARDIVLVSAITLFFFLLALRIPQRLCQRLRIRLPHRYQRLRTED

The top scoring sequence with a 100% identity from the BLAST™ database was that for human herpes virus 5 strain Towne UL16 gene, complete CDS (GenBank accession No. AY297445.1). This sequence was codon optimized for *Lactobacillus delbrueckii* subsp.

bulgaricus using the JCat codon optimization tool and returned the following cDNA sequence:

atggaacgccgccgcggcactgttccactgggctgggttttcttcgttctgtgcctgtcagcttcatcaccatgcgctgttgacctgggctcaa
agtcatcaaactcaacttgccgcctgaacgttactgaactggcttcaatccacccaggcgaaacttggactctgcacggcatgtgcatctcaa
tctgctactacgaaaacgttactgaagacgaaatcatcggcgttgctttcacttggcaacacaacgaatcagttgttgacctgtggctgtacca
aaacgacactgttatccgcaacttctcagacatcactactaacatcctgcaagacggcctgaagatgcgcactgttccagttactaagctgtac
acttcacgcatggttactaacctgactgttggccgctacgactgcctgcgctgcgaaaacggcactatgaagatcatcgaacgcctgtacgtt
cgcctgggctcactgtacccacgcccaccaggctcaggcctggctaagcacccatcagtttcagctgacgaagaactgtcagctactctgg
ctcgcgacatcgttctggtttcagctatcactctgttcttcttcctgctggctctgcgcatcccacaacgcctgtgccaacgcctgcgcatccgc
ctgccacaccgctaccaacgcctgcgcactgaagactaa

This cDNA sequence driven by a separate T7 promoter was inserted into the plasmidpLEM415-ldhl-NK2-link-USP45 so as to impart resistance to the yogurt bacteria against the expressed NK-2. The two genes were separated by a 1 kB non-coding sequence to minimize expression interference. The final plasmid construct was designated pLEM415-ldhl-NK2-link-USP45-T7-UL16. A map of the plasmid and its sequence can be found in supplementary file 1.

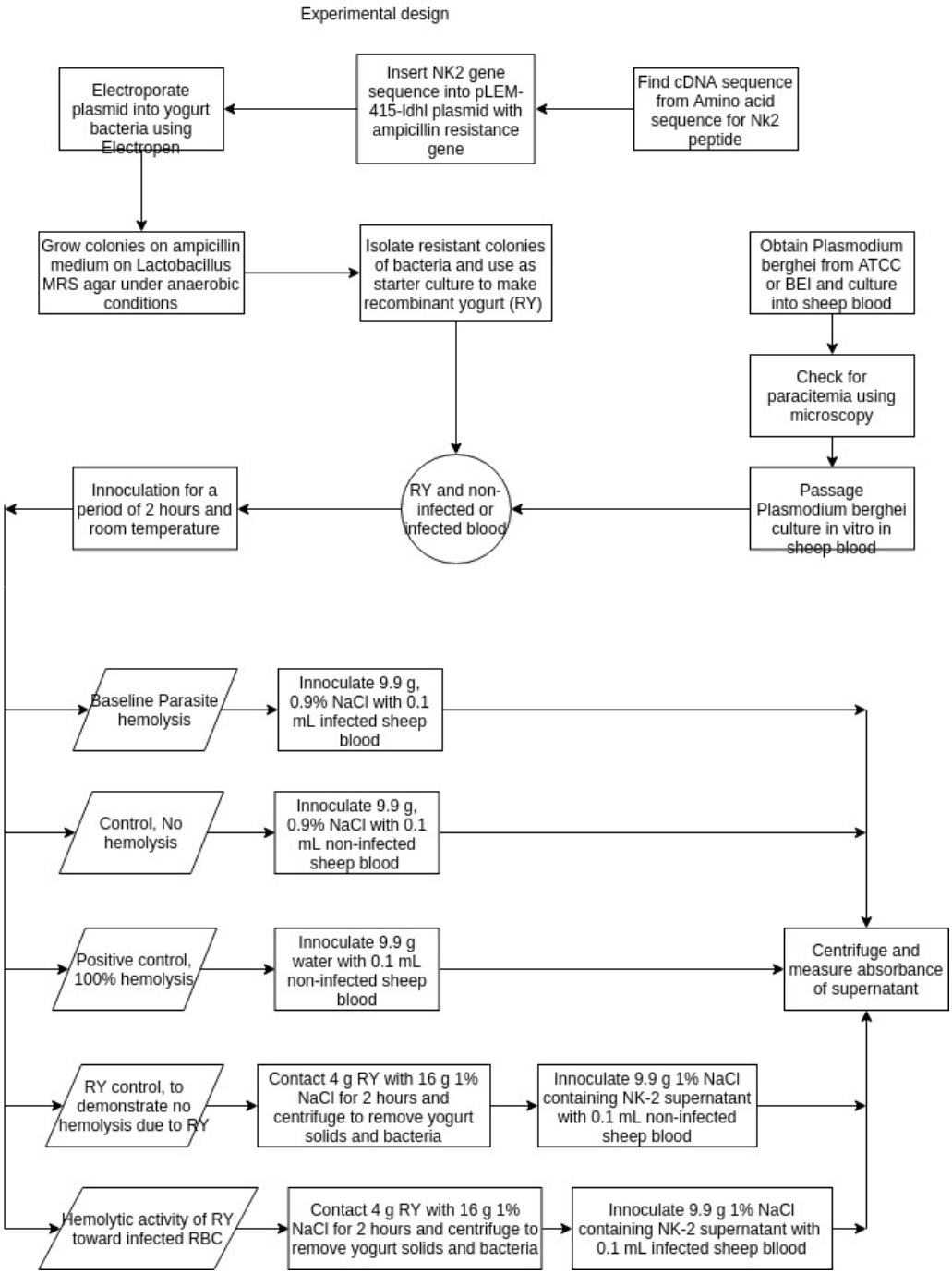
Testing

Defibrinated blood was stored refrigerated at 4 to 8° Celsius and used within 3 months of receipt. All solutions and supernatants containing hemoglobin were diluted with water such that the absorbance was between 0.1 and 2.0 at wavelengths of 540 nm and 580 nm. These two wavelengths were chosen because the literature indicated maximum absorbance at these wavelengths (64). The absorbance at these wavelengths is sensitive to pO_2 levels (65), however these were unchanged during the duration of the experiment. Hemoglobin concentrations were compared at equivalent dilutions. 0.9% and 1% sodium chloride solutions were prepared in volumetric flasks. Typically, for sample nos. 1, 2 and 4, 1 mL blood was pipetted into a culture tube and contacted with either 9 g water, 9 g 0.9% sodium chloride solution or 2 grams of yogurt and 7 g of 1% sodium chloride solution for a period of one hour at room temperature. After one hour, all samples were centrifuged at a relative centrifugal force (RCF) of 249 g for 10 minutes. The cells or cell debris and the yogurt solids settled out at this RCF leaving a clear supernatant which could be analyzed for hemoglobin content by spectrophotometry. Samples 3 and 5 were treated differently in that the yogurt solids – but not the yogurt bacteria – were precipitated out before contacting the resultant diluted clear yogurt aliquot with blood. Briefly, for samples 3 and 5, 4 g of yogurt was mixed with 14 g of either 0.9% or 1.0% sodium chloride solution (samples 3 and 5 respectively). These were then centrifuged at an RCF of 249 g for 10 minutes. 9 g of the clear supernatant was added to 1 mL of blood and stored at RT for 1 hour. After one hour, the samples were centrifuged at an RCF of 249 g for 10 minutes and the clear supernatant was analyzed for hemoglobin content after suitable dilution as above.

Statistical analysis

Independent two-tailed unpaired Student t tests were used to assess the statistical difference between the different samples from one another and from the control (water) (66). Differences between means were considered significant at a p-value < 0.05.

Figure 2: Experimental protocol



Results and discussion:

The pH and conductivity of the distilled water was 6.7 and 7.5 µS cm^{-1}. These values were similar to those measured for store bought water (refreshe™, purified drinking water, Better living brands LLC, Pleasanton, CA) at 6.8 and 7.5 µS cm^{-1} respectively.

Table 1 shows the percent hemolysis for various sample preparations used in the study.

Table 1: Hemolysis of Sheep Blood with Yogurt (see methods for sample preparation)

Sample No.	Sample I.D	PERCENT HEMOLYSIS ± SD	
		540 nm	580 nm
1	Distilled water	100	100
2	0.9% saline	16.2 ±1.59	15.3 ±0.698
3	0.9% NaCl solution with yogurtprecipitated out	46.7 ±1.9	46.9 ±1.15
4	1% NaCl with yogurt not precipitated out	45.6 ±3.70	49.4 ±6.15
5	1% NaCl with yogurt precipitated out	22.5 ±6.70	20.2 ±5.15

n=3 for sample 2, n=2 for all other samples

Baseline hemolysis of sheep blood when diluted with 0.9% sodium chloride solution (sample 2) was 16.2% and 15.3% at wavelengths 540 and 580 nm respectively Hemolysis. Addition of water completely hemolyzed the red blood cells (sample 1) and was used as the positive control.

Table 2: Calculation of osmolality of yogurt

	Weight (mg)	Moles	Molal concentration	Osmolal concentration
Sodium	60	0.001	0.0056	.0112
Sugar	6000	0.0175	0.116	0.116
Calcium	150	0.002	0.013	0.026
Potassium	170	0.002	0.013	0.026

From the nutritional labeling for the osmotic pressure contributing components of the yogurt in 5.3 ounces (150.2 g) (67), the total osmolality of the yogurt was calculated to be 179 mOsm/Kg. It was assumed that the cations were present as their chlorides, hence the osmolal concentrations are twice their molal concentrations. The moles of sugars were calculated as sucrose equivalent. For the mixture of yogurt and NaCl to be isotonic with

blood, the following calculation was performed:

$$(2g * 179\,mOSm/kg) + (8g * x)/10 = 308\,mOSm/kg \quad \text{Equation 1}$$

Where x is the osmolality of the sodium chloride solution such that a mixture of 8 parts by weight of the sodium chloride solution when mixed with 2 parts by weight of yogurt is isotonic with blood. The value of x was calculated to be 340.25 mOsm/Kg, equivalent to 0.99% w/w of sodium chloride solution. Consequently, a 1% solution of sodium chloride was used in experiments corresponding to samples 4 and 5.

Sample 5 showed significantly lesser hemolysis than sample 3. Since the only difference between these two samples was the osmolality of the sodium chloride, it was concluded that the osmolality of the sodium chloride in the presence of yogurt (at the ratio added) did need to be adjusted per the calculations above in order to minimize hemolysis. Sample 5 also showed significantly less hemolysis than sample 4. The yogurt solids therefore contributed significantly to hemolysis.

Bacteria are generally harvested as a centrifuged pellet at an order of magnitude RCF of 10^3 g for~ 10 minutes (19). Since the RCF used in this study was an order of magnitude less, it was reasonable to assume that bacterial settling out of supernatant was insignificant so that the measurable (although not statistically different, $p > 0.17$) difference between hemolysis for the means of samples 5 and 2 could be attributed to the hemolytic effect of yogurt bacteria. *Streptococcus thermophilus* is known to cause α-hemolysis of red blood cells on blood-agar by the oxidation of hemoglobin by bacterially produced H_2O_2 (68). On the other hand, *Lactobacillus* strains were generally non-hemolytic on blood-agar (69, 70).

Conclusion

A pLEM415-ldhl-NK2-link-USP45-T7-UL16 plasmid can be constructed and electroporated into yogurt bacteria. The cloning of the UL16 human herpes virus 5 cDNA imparts resistance to the RY against expressed NK2. Bacteria that have been transformed to express NK-2 can be isolated from colonies grown on ampicillin MRS agar and used as a starter culture to make RY containing ~ 10^{10} CFU. The expressed NK-2 from RY can be recovered by centrifugation at RCF > 10^3 g to exclude both yogurt solids and yogurt bacteria. The NK-2 can then be mixed with sheep blood previously infected and cultured *in vitro* with *Plasmodium berghei* to evaluate the antiparasitic properties and efficacy of the RY.

An adult would need to ingest two 5.3 ounce servings of RY per day to achieve a therapeutic concentration of NK-2 in blood. The expression of heterologous proteins from bacteria transformed with plasmid vectors is notoriously difficult to predict and especially so for a multi-gene expression cassette such as the one described here. The problem of getting lactic acid bacteria to express heterologous proteins that cross into systemic circulation is non-trivial but not insurmountable. If successful,

such genetically engineered GRAS certified yogurt bacteria could be made to express a host of beneficial proteins, orally delivered, 'manufactured' at-site, lending a new meaning to the word 'probiotic' and ushering in an era of 'open source' medicine.

Acknowledgements

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