Isolation, Identification, and Characterization of Small Bioactive Peptides from Lactobacillus GG Conditional Media that Exert Anti-Bacterial Activity

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Background

- Diarrheal diseases remain a major human plague that still claim millions of lives every year.
- Probiotics are non-pathogenic bacteria that are claimed to have several beneficial effects related to their capability to prevent the growth of several pathogenic microorganisms, including *E. coli*.
- There are several theories proposed to explain the antibacterial effects of probiotics, including their capability to compete for nutrients, the establishment of a microenvironment in which pathogenic microorganisms are not able to survive, or the elaboration of toxins lethal for pathogenic bacteria.
- However, their mechanism of action has not yet been completely established.
- It was previously reported that Lactobacillus GG produces small molecular weight (<1,000 D) substances in their culture media which inhibit a wide range of bacterial species.

AIM

To fully characterize the small molecular weight substances elaborated by LGG that exert anti-bacterial effects

Methods

LGG conditional media (CM) **LGG was cultured in 5 ml MRS Broth, at 37^{0}C, with shaking at 225 rpm overnight. The following day, the cultured mixture was centrifuged at 5,000 x g for 45 minutes, the supernatant is conditional media (CM).**

Heat stability **LGG-CM was heated at 95^0 C for 20 minutes and tested in comparison to not-heated LGG-CM.**

Methods

Ion Exchange Chromatography Anion exchange column (d=1.5cm, L=2.0cm flow rate 0.1ml/min). Equilibrium buffer: Tris-HCl (pH 8.0), Elution buffer: ImmunoPure IgG elution buffer (pH 2.8, PIERCE).

SDS-PAGE: was carried out on Criterion precast gel (4%-20%), using Tris-Glycine-SDS buffer (Bio-Rad) as running buffer at constant 170V for 1 hour. The gel was stained by 2.5% Coomassie blue and de-stained by 10% Methonol,7.5% acetic acid solution.

Liquid chromatography/ Mass spectrometry

The conditional media was concentrated, filtered; Liquid chromatography(silica reverse phase C18 column) and MS-MS; Identification analysis against the lactobacillus database (IPI) using SEQUEST search algorithm

Methods

Peptide Synthesis, Purification and Identification

The peptides were synthesized on a Symphony peptide synthesizer using Fmoc coupling strategy;

Peptide purification was performed on a Beckman Gold system consisting of two 110B pumps and a 167 detector (215 nm) using a Dynamax reverse-phase C18 column (8μ, 25.6 x 250 mm);

Peptide characterization was perform by Reverse Phase HPLC and MALDI-TOF.

Activity assay

Measurement of anti-bacterial activity by culture spectrophotometer at A600 10 ul *E. coli* EAEC 042 (7.7 x 1014 CFU/ml) were added to 1.0 ml LB Broth, mixed, and 100 ul of peptides solution dissolved in MRS was added to the mixture. MRS alone (100 ul) or LGG CM 100 ul (LGG concentration was 19.7x1012 CFU/ml) were used as negative or positive controls, respectively. The mixture was cultured for 3 hours with shaking at 225 rpm, 37^0C, measuring A600 at the end. The relative inhibition activity was calculated according to the following formula: (1- SampleA600 / Control A600) x100 and expressed in percentage. In selected experiments, antibacterial effects were assayed by colony counting and the relative inhibition activity was calculated according to the following formula: (1- Sample colonies / Control group colonies) x 100 and expressed as percentage.

Effect of LGG culture on E. coli growth

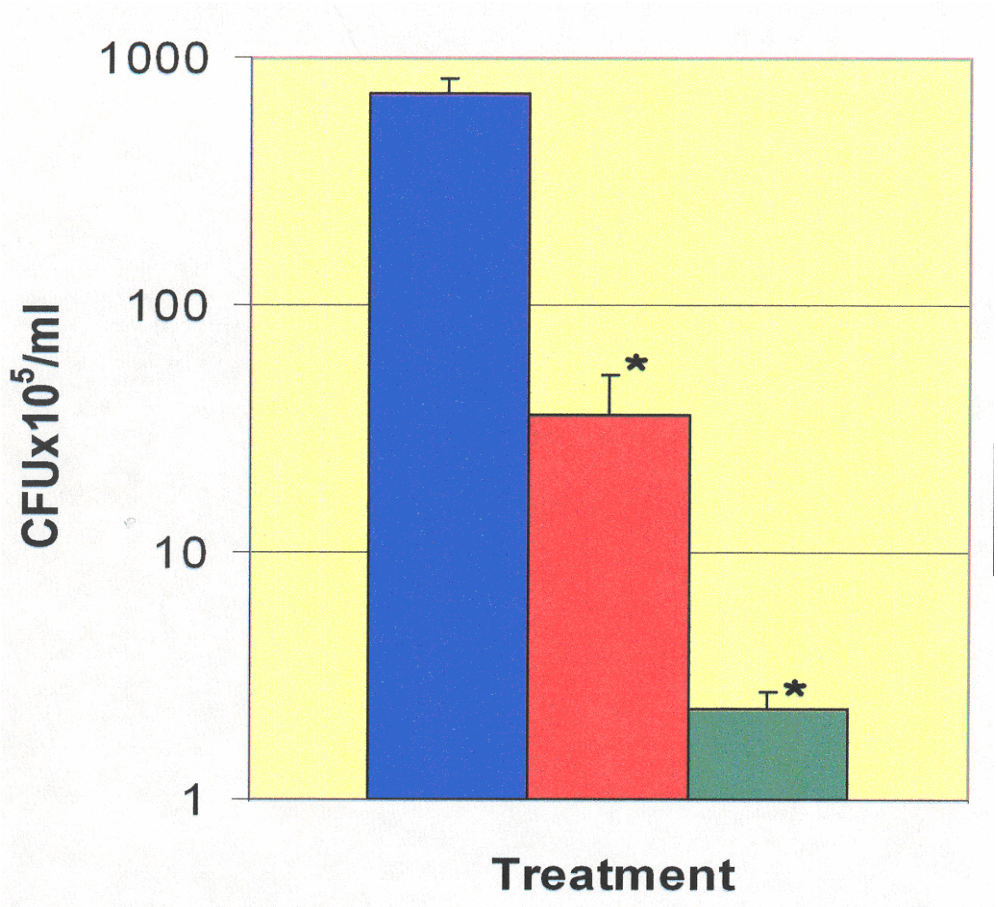

Figure 1. Effect of 100 μl (red bar) and 1000 μl (green bar) of LGG culture on *E.coli* culture survival. *E.coli* only (blue bar) was used as control. P<0.001

Effect of LGG-CM on E. coli growth

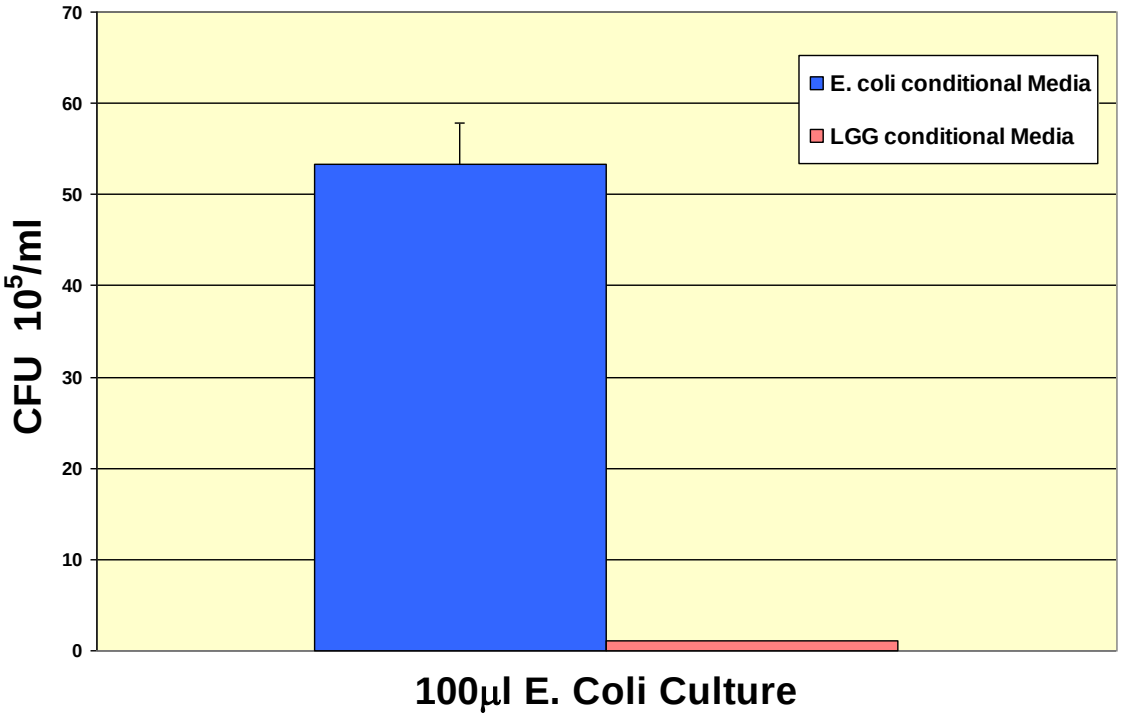

Figure 2. Effect of LGG CM (red bars) *on E.coli* survival. CM from a non-pathogenic *E.coli* was used as a control. *p=0.006; **p=0.0008.

LGG-CM Heat-Stability Experiments

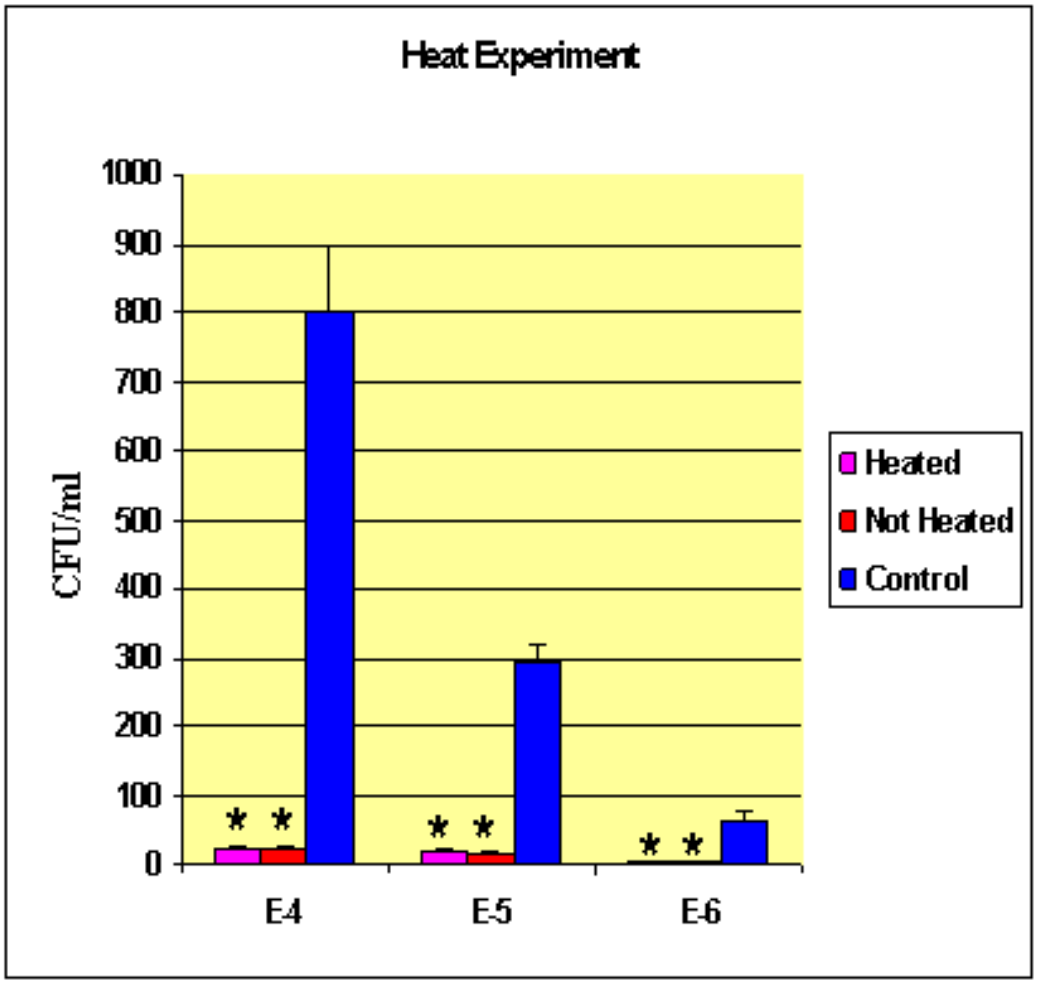

Figure 3. Effect of heat treatment on LGG CM anti-bacterial activity Heat treatment of LGG CM did not affect its anti-bacterial effect, irrespective of the dilution of *E.Coli* 042 cultures. E-x = 10^{-x} dilution *p=. 0.00005.

LGG-CM Ion-Exchange Experiments

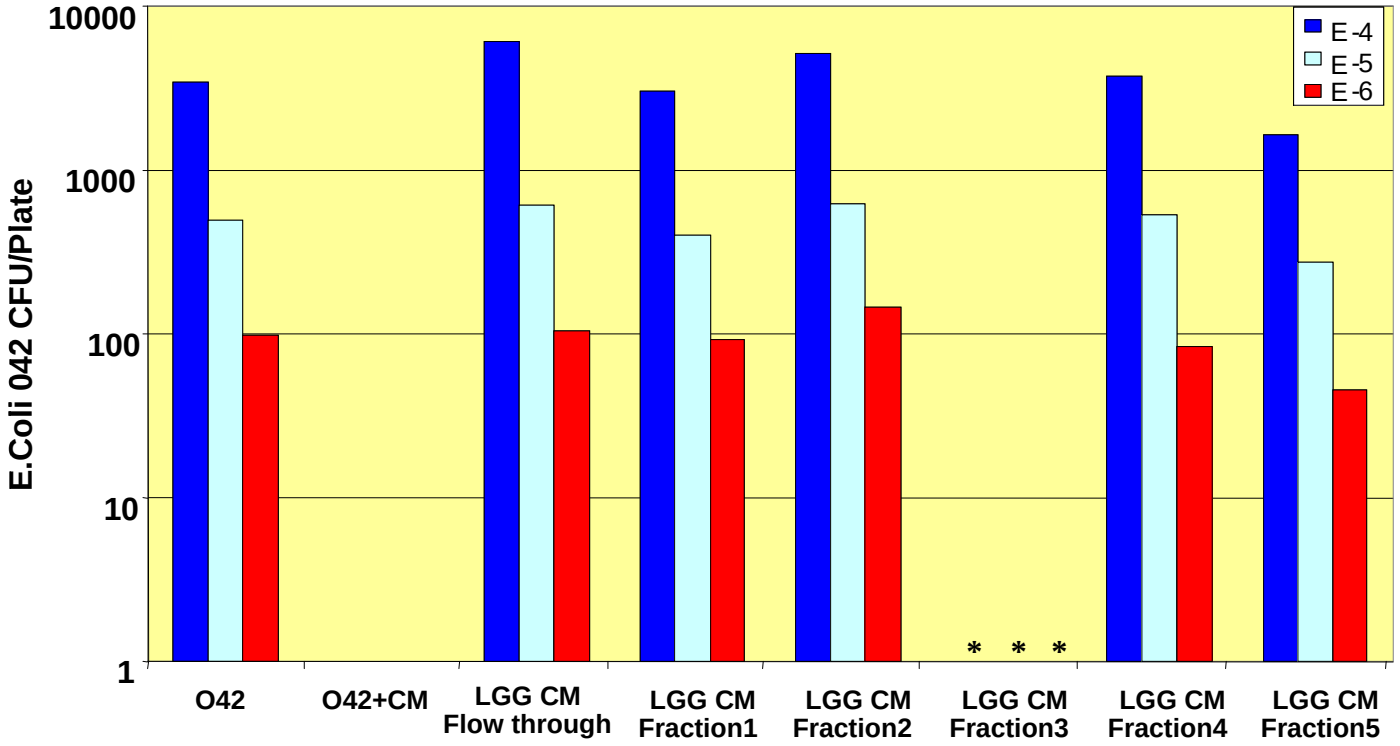

Figure 4.. Effect of LGG CM fractions obtained by Anion Exchange Chromatography on *E.coli* 042 growth. Fraction #3 retained the anti-bacterial effect shown by LGG CM. *p<5x10-7.

LGG-CM LC/MS/MS Analysis

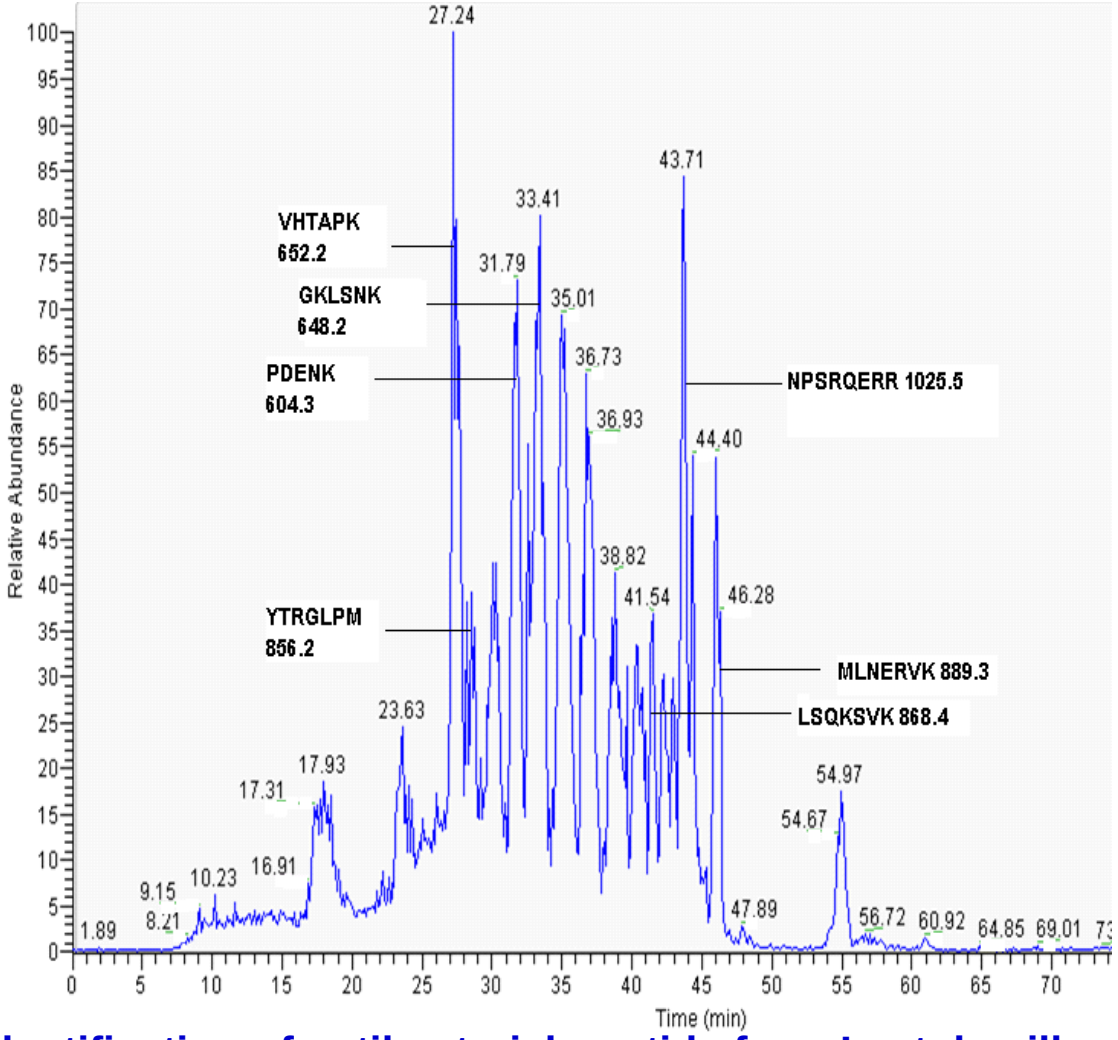

Figure 5. Identification of antibacterial peptide from Lactobacillus GG culture conditional media by LC/MS/MS analysis. The figure shows the typical chromatogram marked with the identified peptides (MS1) by LC/MS and SEQUEST data base search.

Sequences and Characteristics of the Peptides identified in LGG-CM

Of the several fragments of ~ 1,000D molecular weight, the following seven peptides resulted being part of the lactobacillus GG proteome:

	Molecular Weight	PI	Charges
NPSRQERR	1025.5	11.7	+2
PDENK	604.3	4.37	-1
YTRGLPM	856.2	8.75	+1
VHTAPK	852.2	8.73	+2
LSQKSVK	868.4	10.0	+2
MLNERVK	889.3	8.5	+1
GKLSNK	648.2	10.0	+2

E.Coli growth Time Course

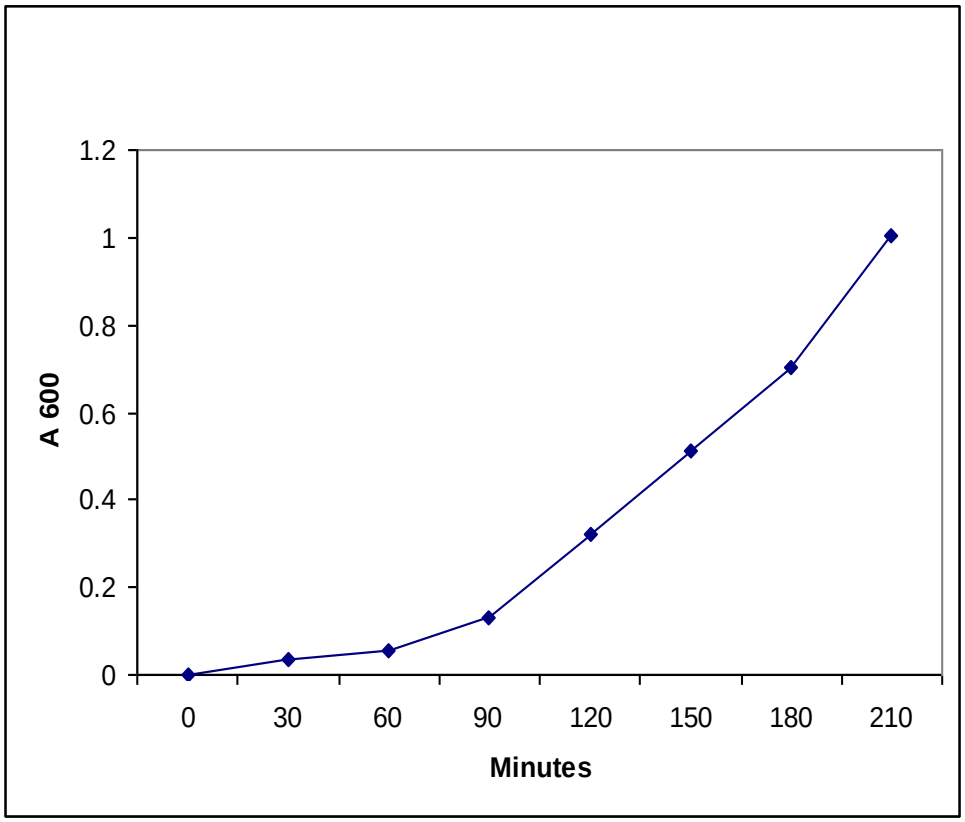

Fig 6A. E. coli growth time-course. E. coli shows a liner growth between 90 minutes and 210 minutes.

Anti-Bacterial Effect of the 7 Identified LGG-CM peptides

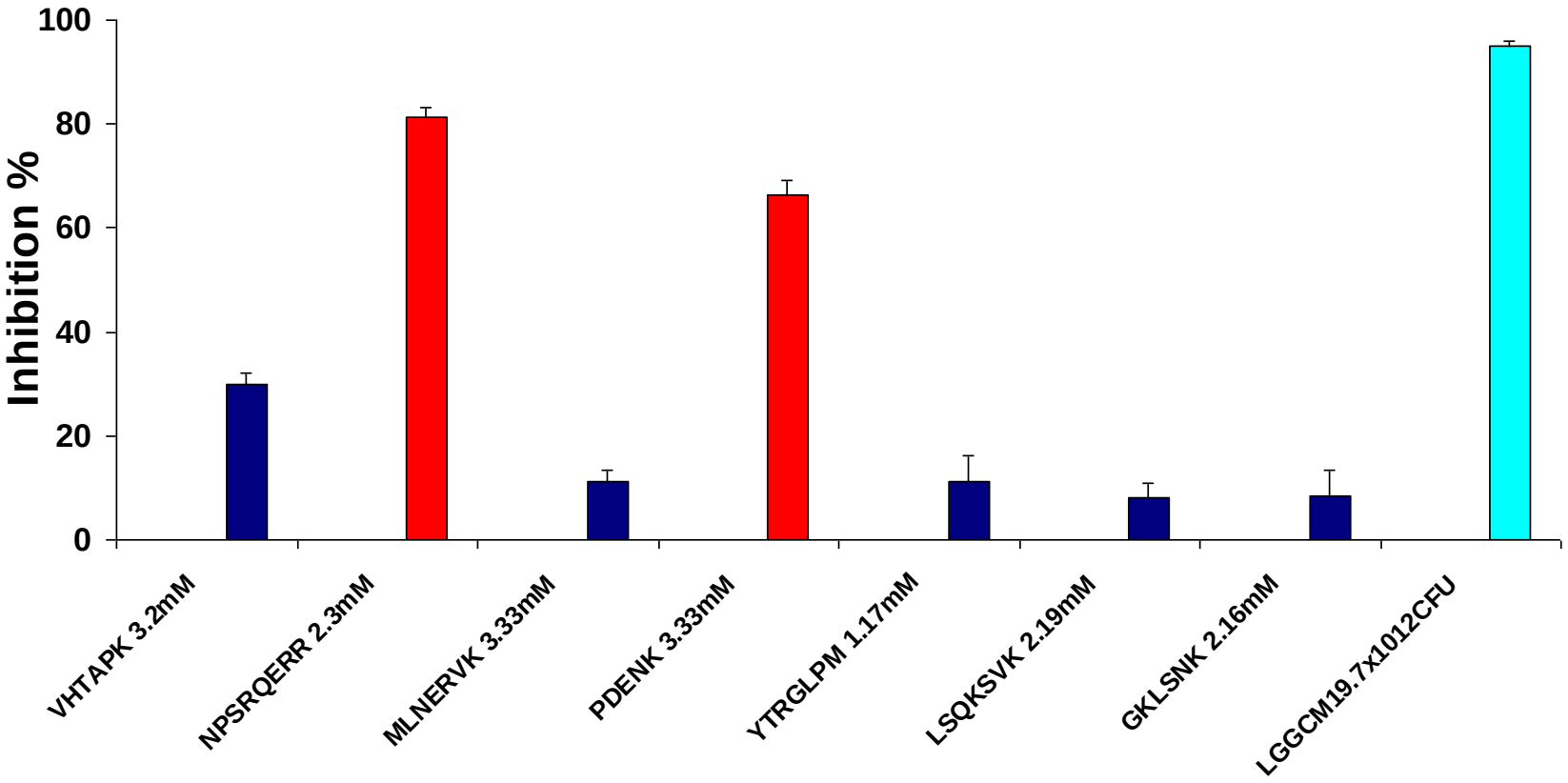

Fig 6B. Peptide NPSRQERR, PDENK, VHTAPK, MLNERVK, YTRGLPM, GKLSNK , LSQKSVK and LGGCM(19.7x 1012 CFU/ml) relative inhibition activities on E.coli growth. Figure shows mean + SD, n=3 except LGGCM n=7

Different Effects of NPSRQERR on Gram-Negative and Gram-Positive Bacteria

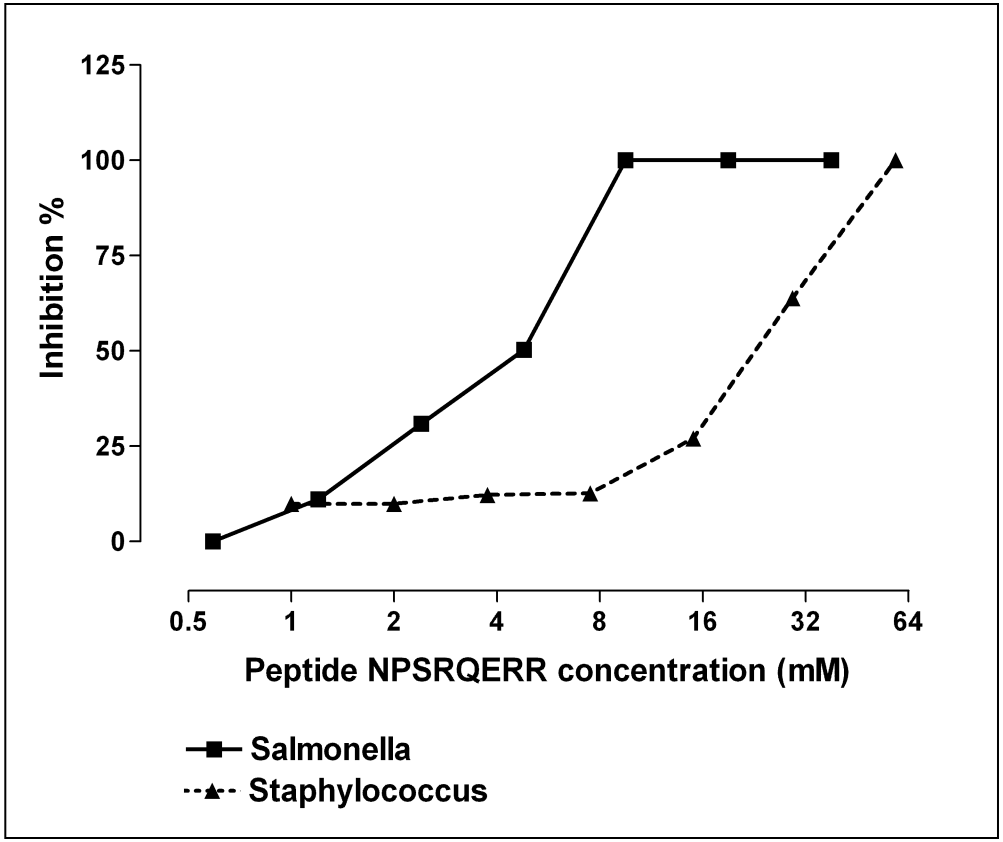

Fig 7. Peptide NPSRQERR Inhibition activities on Salmonella and Staphylococcus growth. The effect on Salmonella more than on Staphylococcus.

DISCUSSION

- **Our results concerning small peptides elaborated by LGG may open unexplored horizons for effective alternative treatments of diarrheal diseases.**

- **The use of a small peptide as an anti-diarrheal drug is novel and would offer several advantages over current treatments.**

Comparison between LGG peptide and other antimicrobial peptide

	LGG peptides	Antimicrobial peptides
Peptide in length	5 – 8 amino acid	12 – 50 amino acid
Secondary structure	No	To form secondary structure
Hydrophobic residue	Not necessary	> 50%
With positive charge or negative charge	Yes	Yes
Anti- Gram positive and/or negative bacteria	Yes	Yes
Anti-viruses, fungi or cancerous cells	Do not know	Yes
Function as immunomodulator	Do not know	Yes
To kill antibiotics resistant strain	Do not know	Yes

Conclusions

- **LGG elaborates at least two small peptides showing various degrees of anti-bacterial activity.**

- **Peptide NPSRQERR showed the most potent anti-bacterial effect that was detected both on Gram-negative and Gram-positive microorganisms.**

DDW 2008 Disclosure

The following authors have no financial disclosure to declare

1. Ruiliang Lu: Mucosal Biology Research Center, University of Maryland School of Medicine
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The following authors have financial disclosure to declare

1. Alessio Fasano: Mucosal Biology Research Center, University of Maryland School of Medicine: Stock Holder Alba Therapeutics