Report STSM

Active edible packaging with Antimicrobial Random Peptides as food preservative

1.Introduction

1.1 Premise

The aim of the STSM was the development of antimicrobial edible packaging using Random peptides as food preservative. This mission was requested by the collaboration between the University of Modena and Reggio Emilia and the Hebrew University of Jerusalem. The STSM period was of two months and the work plan was the following:

1. Synthesis of Antimicrobial Random Peptide (ARPs)
2. Inclusion of ARPs in pectin edible film
3. Test the antimicrobial activity against *E.coli* and *B.subtilis* in vitro
4. Test the Mesophilic aerobic bacteria trend in meat matrix after the application of an antimicrobial coating by Dipping techniques.

1.2. Antimicrobial Random Peptide (ARPs)

Antimicrobial random peptides were used to decrease the mesophilic aerobic bacteria of minced meat. Random peptides are random in terms of sequence, but highly controlled in terms of chain length and stereochemistry. The mechanism of action of synthesized random peptides is similar to the Host-defense peptides (HDPs). HDPs are produced by eukaryotes as part of the innate immune response to bacterial infection. These types of positively charged peptides are rich in hydrophobic residues, which mediate disruptive interactions with the hydrophobic interior of the bacterial membrane lipid bilayer.

1.3. Pectin as polymer to create edible Packaging

Pectin is commercially produced from citrus peel as a by-product from extraction of lime, lemon, orange peels and from apple pomace, the dried residue remaining after extraction of apple juice. Pectin is a heteropolysaccharide in its native state, but acid extraction removes most of the neutral sugars such as rhamnose, galactose, arabinose, etc., that comprise the branched or “hairy” regions of the polymer. Pectin is used in food mainly for its gelling property, which is influenced by its degree of esterification (DE). Gelation properties also translate into better interpolymer chain associations and film formation; therefore, pectins will generally form stronger films when gelation conditions (pH and/or presence of calcium) are met. Pectin films are generally not as strong as alginate films. Methyl substitution (a larger group than –COOH) and the presence of some remaining “hairy regions” or branching on pectin molecules seem to pose more steric hindrance to film formation than the smaller uronic acid groups of alginate.

1.4. Food Matrix

The food Matrix used in this study was Kosher Beef Meat. Kosher, that means ”ritually pure”, meat is the one used from the Jewish population and is still widely practiced Jews. The global volume and value of kosher meat are commercially enormous. More than 37 million USD of kosher red meat is imported all

around the world in 2012. Producers and consumers demand that the quality of this meat is on par or even better than their equivalents produced using conventional methods. Kosher Meat is a large part of the human diet and in many countries a great part of the food industry. Consequently, the safety of Kosher meat is of major concern to consumers and food service industries. The spoilage of raw meat is largely dependent on initial bacterial flora, the amount and types of microorganisms (pathogenic and/or spoilage), as well as on the meat package and storage conditions. Kosher minced beef is considered as an ideal substrate to support the growth of several spoilage and pathogenic bacteria because of its high concentration of nutrients and high water activity. The deteriorative effects caused by bacterial growth are discoloration, off-odors, and slime production. Deteriorative rate depends primarily on the meat composition, the hygienic practices during the grinding and packaging process, and the storage conditions. These factors bring in a reduction of shelf-life. On the other hand, there are serious problems related to foodborne diseases which are caused by consumption of contaminated meat. To Increase the shelf life and reduce the outbreak of foodborne diseases is necessary to find new technological approaches for food preservation.

2. Materials and Methods

2.1. ARPs synthesis

Peptides are synthesized by coupling the carboxyl group of one amino acid to the amino group of another amino acid molecule. Due to the possibility of unintended reactions, protecting groups are usually necessary. Chemical peptide synthesis most commonly starts at the carboxyl end of the peptide, and proceeds toward the amino-terminus. This is the opposite direction of protein biosynthesis. Solid-phase peptide synthesis (SPPS), pioneered by Robert Bruce Merrifield, caused a paradigm shift within the peptide synthesis community, and it is now the standard method for synthesizing peptides and proteins in the lab. SPPS allows for the synthesis of natural peptides which are difficult to express in bacteria, the incorporation of unnatural amino acids, peptide/protein backbone modification, and the synthesis of D-proteins, which consist of D-amino acids. Small porous beads are treated with functional units ('linkers') on which peptide chains can be built. The peptide will remain covalently attached to the bead until cleaved from it by a reagent such as anhydrous hydrogen fluoride or trifluoroacetic acid. The peptide is thus 'immobilized' on the solid-phase and can be retained during a filtration process while liquid-phase reagents and by-products of synthesis are flushed away.

The general principle of SPPS is one of repeated cycles of deprotection-wash-coupling-wash. The free N-terminal amine of a solid-phase attached peptide is coupled to a single N-protected amino acid unit. This unit is then deprotected, revealing a new N-terminal amine to which a further amino acid may be attached. The superiority of this technique partially lies in the ability to perform wash cycles after each reaction, removing excess reagent with all of the growing peptide of interest remaining covalently attached to the insoluble resin. The amino acids used in this study were:

- Tryptophan (W) and Lysine (K) to create 20-mer WK peptides
- Phenylalanine (F), Leucine (L)) and Lysine (K) to create 20-mer FLK peptides

2.2 Film Inhibition Test (FIT)

FIT was used to investigate the antimicrobial power of the pectin-based polymer. The film was created using a solvent casting technique of pectin gel on Petri dish. The gel was created adding pectin into a solvent. The tested solvents were distilled water added with different concentrations of ARPs. Pectin was added while stirring and heating. Then, 2 mL of gel were poured on a 30 mm Petri dish, and calcium chloride solution were sprayed over the gel for each dish. The drying process was carried out at room temperature for 24 h inside a microbiological sterile hood. The dried films were separated from their support and utilized as disks for Kirby-Bauer test. The FIT was tested against *E.coli* and *B.subtilis.*

2.3 Study of MAB trend in Kosher Meat

The minced meat was purchased from a local butcher in Israel. The meat was a selection of beef's sirloin steak, minced with just washed and disinfected meat grinder. Inside a sterile hood, 200 g of minced meat was mixed with FLK peptide and other 200 g of meat with WK peptide. The peptides were dissolved. The treated minced meat was divided in meatballs of 10 g and put inside a sterile polystyrene tray, wrapped with polyethylene film. The meat was stored inside the fridge at 4°C. Each day of analysis, 3 meatballs for each kind of thesis are analyzed.

The counting of Mesophilic aerobic bacteria (MAB) was determined respecting the microbiological protocol: ISO 4833. Inside a sterile blender bag, the sample (10 g) was mixed with 90 mL of sterile physiological solution using a stomacher, the solution formed after fold serial dilutions were inoculated inside BHIA media. After 24 h of incubation at 30 °C, the bacterial colonies were counted to determine the CFU/g of mesophilic aerobic bacteria.

3. Results

3.1 ARPs synthesis

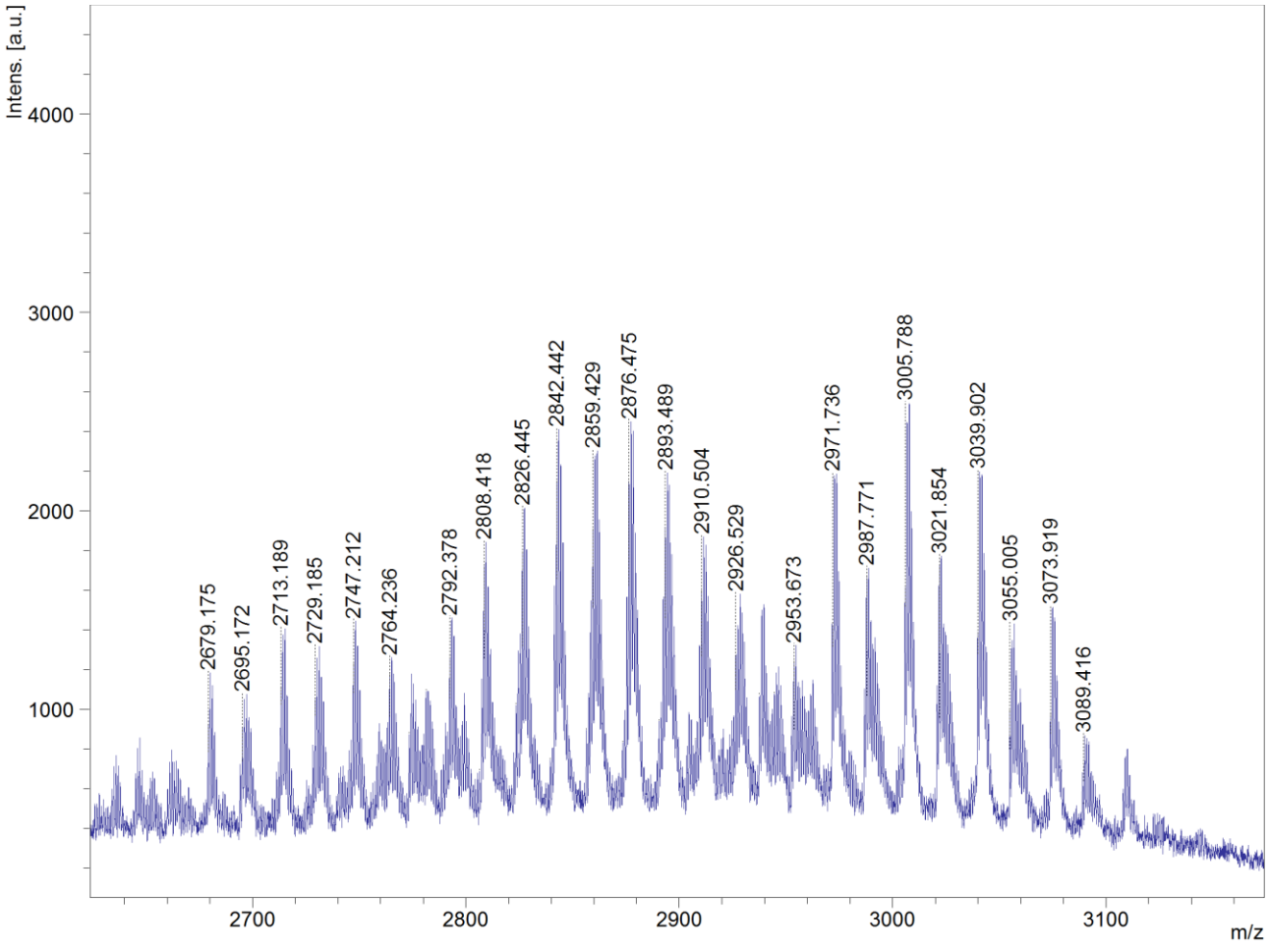

Fig.1. Analysis of FLK random peptides with MALDI-TOF

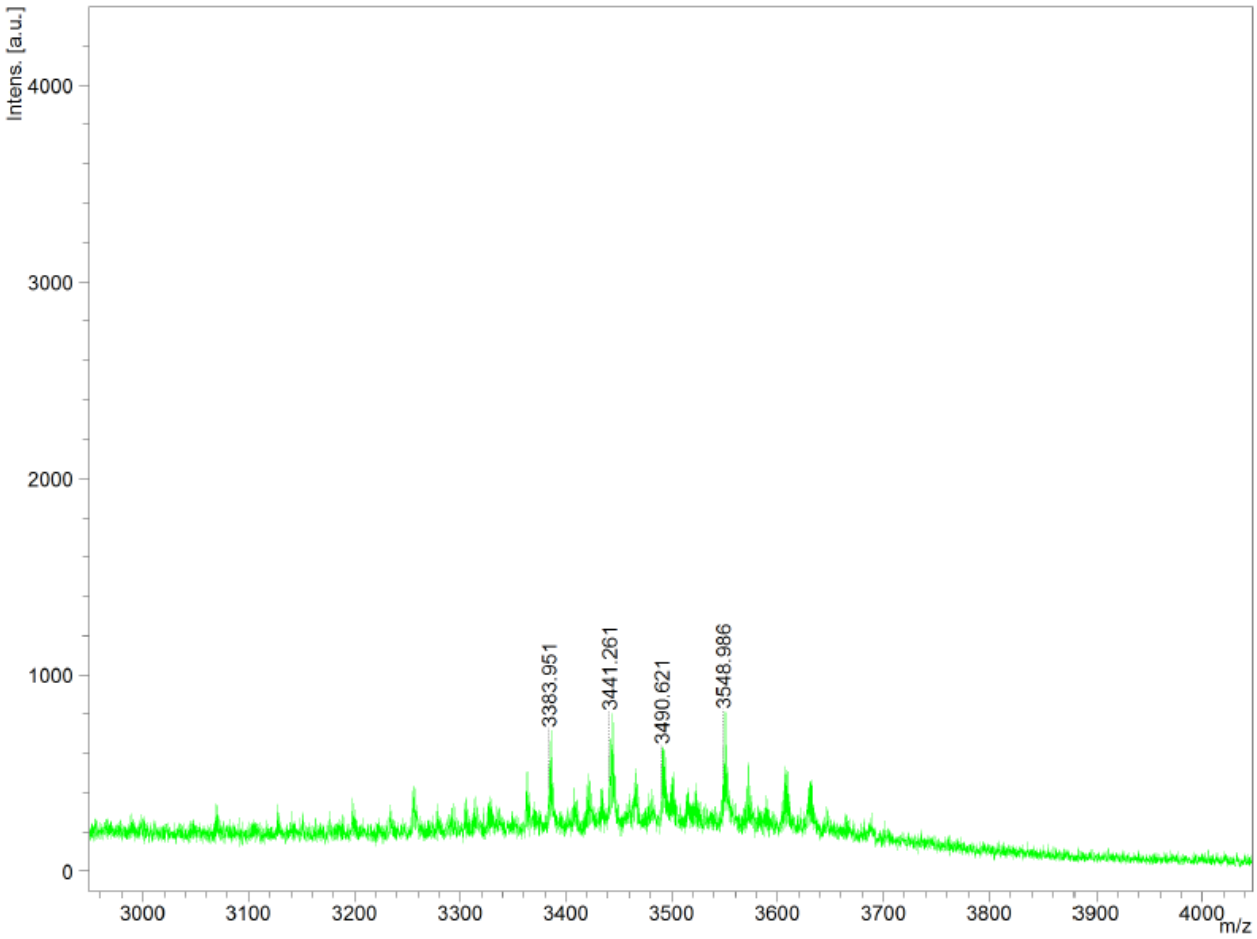

Fig.2. Analysis of WK random peptides with MALDI-TOF

After the synthesis of WK and FLK peptides, these compounds were analyzed with a MALDI-TOF instrument to check the mass of the random peptides.

The Range of the mass are correct for both peptides. The higher number of peaks in FLK chart was due to the composition of the peptides. FLK had 3 amino acids so the possible combination during a Random synthesis were more.

3.2 Film Inhibition Test (FIT)

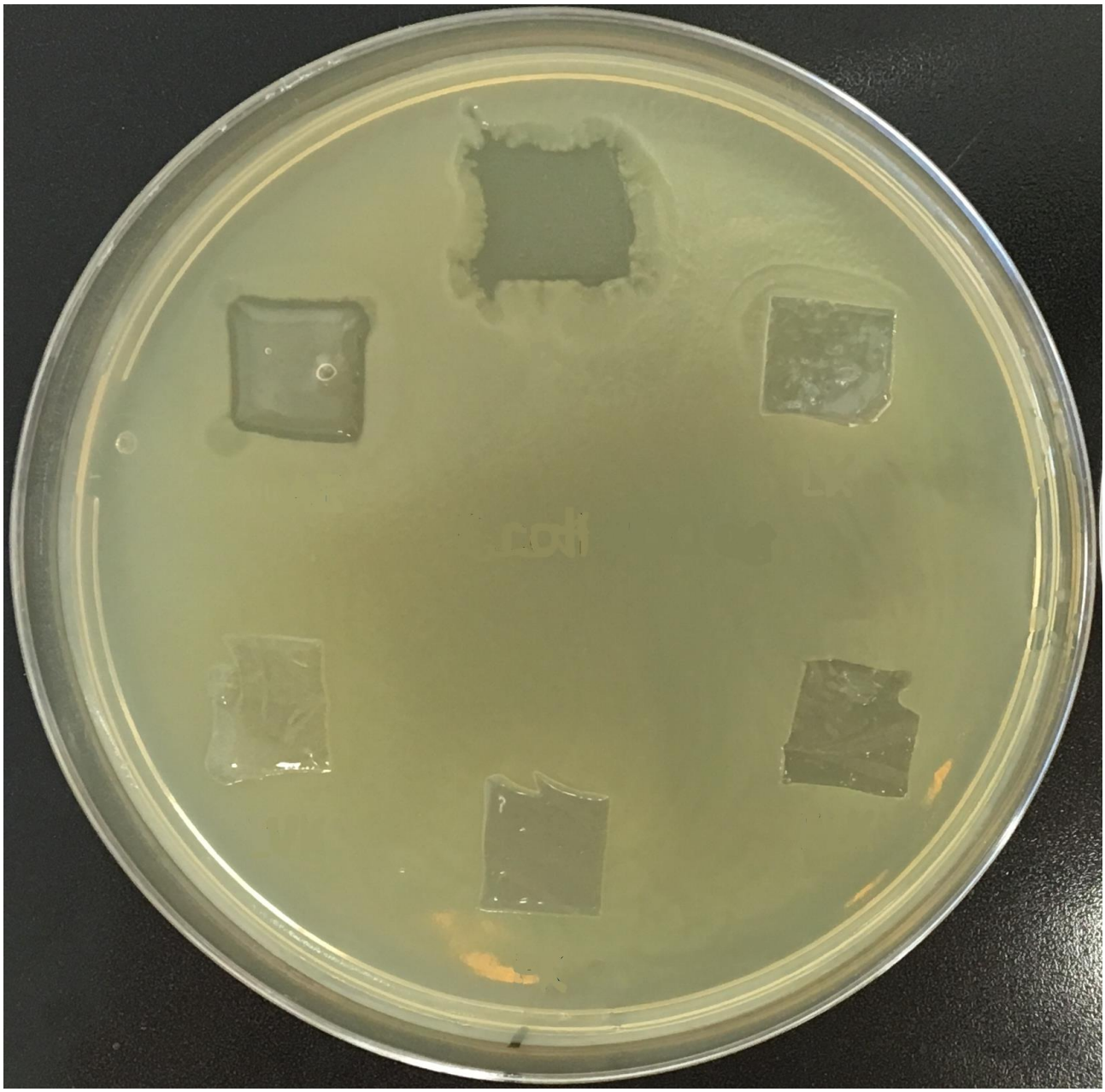

Fig.3. Film inhibition test of WK and FLK pectin film against *E.coli*

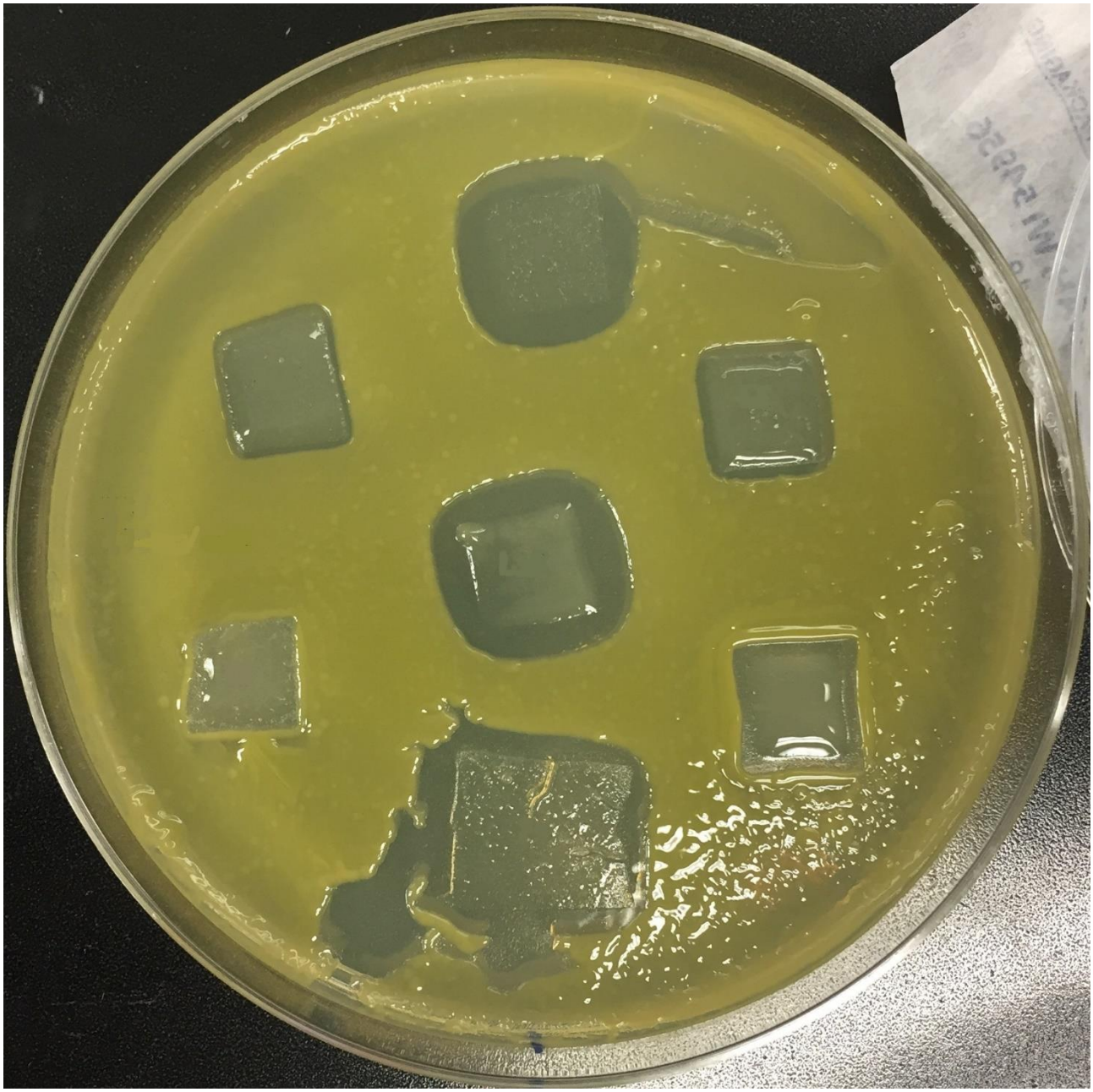

Fig.4. Film inhibition test of WK and FLK pectin film against *B.subtilis*

It is possible to observe how the ARPs included inside edible pectin film can inhibit by contact the growth of *E.coli* and *B.subtilis.* In some case where the ARPs concentration was higher, we obtained an inhibition zone by diffusion as well. It is possible to say that the new formulation of active edible packaging showed a great antimicrobial activity.

3.3 Study of MAB trend in Kosher Meat

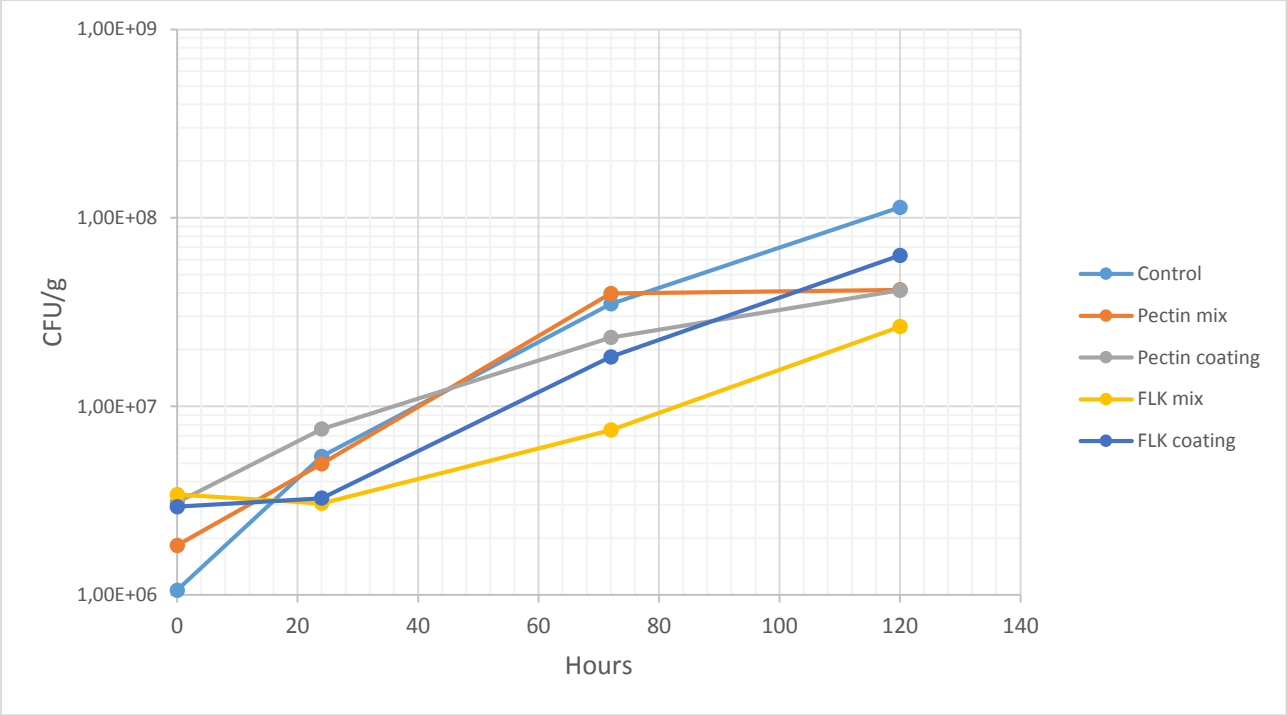

Fig.5. MAB trend of kosher meat after the treatment of FLK e/o Pectin film (applied by dipping techniques)

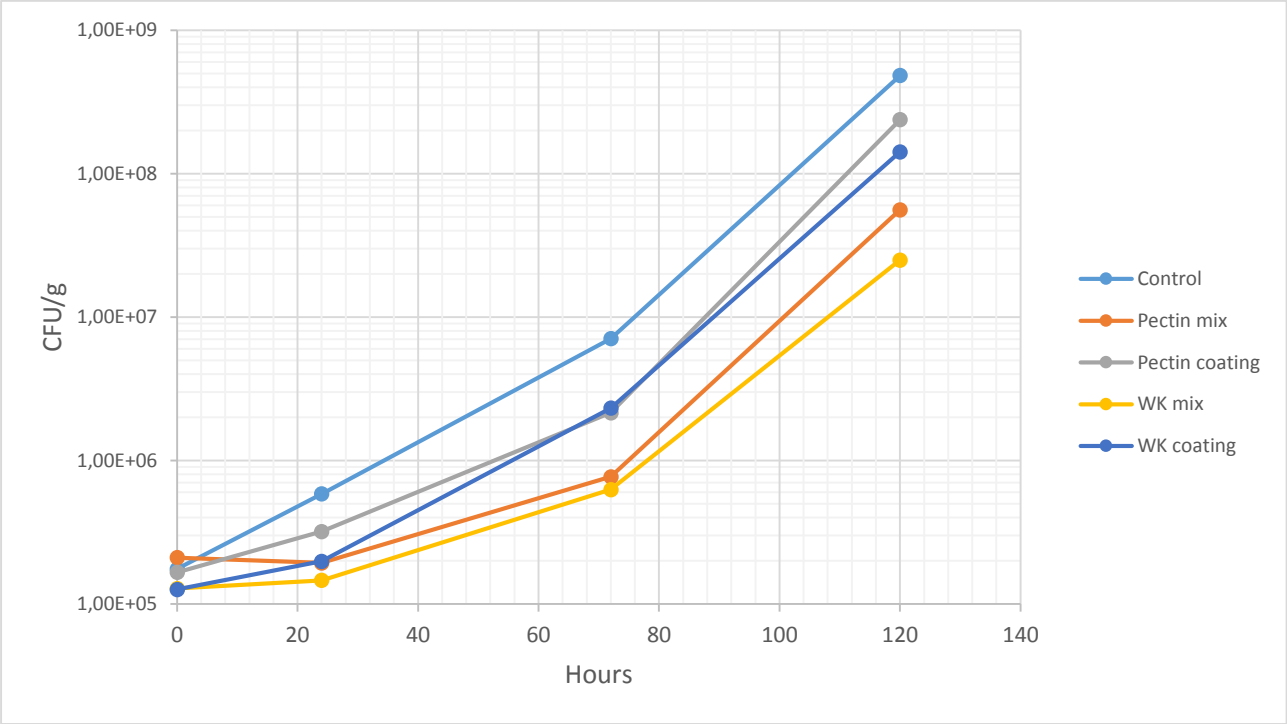

Fig.6. MAB trend of Kosher meat after the treatment of WK e/o Pectin film (applied by dipping techniques)**.**

It is possible to observe 1 log reduction in the MAB growth in all the sample treated with coating and ARPs. The quantity of ARPs used in these studies was very low, 5 mg/mL of Film forming solution of pectin. The inclusion of so little quantity inside a pectin polymer can explain why the Antimicrobial activity was better when ARPs were mixed directly with the meat.

Conclusion

To conclude, it is possible to say that these novel active edible films can reduce the spoiling bacterial population on Kosher meat increasing the shelf-life and the food safety. Only one kind of edible polymer was used, so in the next future it will be interesting to see the behavior of ARPs inside other kinds of edible packaging. On the other hand, it will be interesting to study the MAB trend using a higher concentration of ARPs inside the edible film and test it on other food matrixes as well.

Acknowledgments

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