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1. Executive Summary

NanoPack will develop and demonstrate a solution for extending food shelf life by using novel antimicrobial surfaces, applied in active food packaging products. It will develop, scale up and run pilot lines in operational industrial environments to manufacture antimicrobial polymer films that are commercially feasible and accepted by retailers and consumers alike.

To fully achieve these goals, an assessment plan for testing and characterizing the antimicrobial activity of NanoPack films has been designed. This plan specifies the microorganisms (both bacteria and fungi) that will be used as well the relevant food matrices.

2. Model microorganism for foodborne pathogen

Packaging conditions heavily influence the growth of spoilage microorganisms that are naturally present in the food product and play a role in selecting microbial species. For this reason, the model microorganisms to be used in the project should represent the common foodborne pathogens present, as this will affect the antimicrobial characterization of all developed materials.

Thanks to the cooperation between technical and industrial partners, the target microorganisms have been identified and selected. The choice of microorganisms is based on the following considerations:

1. Types of food to be packaged following the consortium's partners and needs.

2. Complying with proper safety considerations, as NanoPack trials will be based on relevant EU legislation and Food Safety Authorities guidelines.

The chosen model microorganisms will be used for all antimicrobial tests throughout the project duration. Initially, antibacterial activity of the different samples will be evaluated using the **Kirby-Bauer technique.** The assessment will record the zone of bacterial inhibition around the circumference of a polymeric sample disc.

In addition, quantitative growth in liquid media will be studied using **the Japanese standard** (JIS Z 2801: 2000 "Antimicrobial products – test for antimicrobial activity and efficacy").

To investigate whether the antimicrobial activity of studied samples can be achieved without a direct contact (i.e., in headspace) between a polymeric film and the model microorganism, **the micro atmosphere test** will be employed. This technique relies on enclosing microorganisms and polymeric specimens in a sterile container without a direct contact between them (e.g., inoculating agar in a Petri dish with a microorganism and attaching a polymer film to the lid of the Petri dish). The antimicrobial activity of the studied films can then be the represented as a zone of bacterial inhibition (in case of bacteria) or through kinetics of fungal colony growth (in case of fungi). Detailed protocols of these assays are attached as Appendix 1 to this report.

These antimicrobial assays will be utilized by the TECHNION for testing antimicrobial activity of polymer films. These assays will be also followed by all other partners, who wish to reproduce the results or carryout additional studies.

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Table 1. The selection of the target microorganisms

Food product	Target microorganisms in the development phase	Target microorganisms in the optimization phase
Meat product	**Gram negative**: *Escherichia coli* **Gram positive**: *Listeria innocua*	Aerobic colony count **Gram negative**: *Pseudomonas* *Enterobacteriaceae* *Salmonella* *Escherichia coli* **Gram positive**: *Lactobacillus* *Staphylococcus aureus* *Listeria* **Yeast & Molds**
Bread	**Molds** *Penicillium*	**Molds** *Aspergillus* *Penicillium*
Cheese	**Gram negative**: *Escherichia coli* **Gram positive**: *Listeria innocua*	**Gram negative**: *Enterobacteriaceae* *Salmonella* **Gram positive**: *Staphylococcus aureus*
Fresh Salmon	**Gram negative**: *Escherichia coli*	

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	Gram positive: *Listeria innocua*	
F&V	**Gram negative**: *Escherichia coli* **Gram positive**: *Listeria innocua*	

By using *E.coli* & *Listeria* as target microorganisms in the development phase, we could assess the efficiency of the antimicrobials against Gram+ and Gram- bacteria. *E. coli* has been included, since it is similar to *Salmonella* (with respect to cell structure), but easier to handle (e.g. in the Japanese standard test).

3. Preliminary testing

Preliminary testing of existing lab-scale packaging material on several food products by each food producer will be done to determine the best candidate food items, best packaging types and proper environment for maximum benefit with NanoPack packaging compared with control. The preliminary testing protocols will be developed at the pilot plants of CTIC-CITA, where small batches of food products will be tested.

The shelf life of the food products is limited by either microbiological or sensory degradation. These two factors will be monitored by microbiological and organoleptic testing over the products shelf life to ensure product safety.

- **Microbiological assays:**

To understand the growth profile or inhibition of significantly high risk pathogens over the proposed storage life. Inoculated and analysed in CTIC-CITA laboratory to understand the growth parameters of specific pathogenic bacteria of interest for the food product.

- **Physicochemical assays:**

CTIC-CITA will carry out product characterization analysis (quality) based on the main physicochemical characteristics: colour, texture, pH, water activity (a_w).

- **Sensorial analysis:**

To assess the duration in which the product is acceptable organoleptically, and to analyse whether an interaction between the packaging material and the food composition has taken place or not, tests will be conducted by CTIC-CITA technician using a trained panel of assessors. The panel will evaluate each product using qualitative and quantitative descriptive tests (QDA). The differences between tested products vs. control will be evaluated statistically.

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In these preliminary studies, 3 samples of each selected food product will be packaged in antimicrobial film produced by Technion and Carmel in a collaborative work at a lab scale.

Meat products

Dawn Meats supplies a quality range of beef and lamb carcase and primal cuts throughout different brands. Their products are packaged and presented in a range of different styles and packaging formats, ensuring attractive presentation and prolonged shelf life.

Two basic types of meat that will be tested in NanoPack films are:

- Whole muscle: striploin steaks → in vacuum / skin packaged.
- Comminuted muscle: minced → in MAP (modified atmosphere packaging).

Meat samples will be packed using NanoPack films containing bioactive agents. At the same time (as a control experiment), the same food products will be packaged with standard packaging material. Thus, the shelf life assessment for both 'NanoPack Samples' and 'standard samples' will be evaluated and statistically analyzed.

Bakery products

Pão de Gimonde is a Portuguese SME bakery that links tradition and innovation in their product line. The products baked by Pão de Gimonde are mainly typical of the North-eastern region of Portugal, namely wheat bread, rye bread and 'Folar Transmontano' (a traditional bread to which pieces of raw-cured meat sausages are added). Pão de Gimonde's main customers are retail outlets such as supermarkets, small shops and markets in Lisbon, Oporto and throughout the region of Minho.

The company market share is based on wheat bread, rye bread and 'Folar Transmontano', therefore these products will be tested during the NanoPack project. This selection is not only based on the replicability in other companies with similar types of bread, but also the market share.

During the preliminary testing, wheat bread and rye bread are going to be tested in NanoPack film, and for comparison, the same food products will be packaged with standard packaging material. Thus, the shelf life in both 'NanoPack Samples' and 'standard samples' will be evaluated.

Dairy products

Arla Foods is an international dairy company, with a wide range of dairy products of highest quality. Arla Cream Cheese is produced from natural ingredients and is characterized by its distinct fresh taste, therefore this food product will tested during NanoPack project.

Cream cheese will be packed using NanoPack films containing bioactive agents, and for comparison, the same food product will be packaged with standard packaging material. Thus, the shelf life in both 'NanoPack Samples' and 'standard samples' will be evaluated.

Other food matrices

CTIC-CITA will also test other food matrices in NanoPack developed films. Thus, attained results will be used for the business plan.

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Following the interest of some of NanoPack partners, the products which will be tested are:

- Fresh Salmon → Tommen Gram actually commercializes fresh salmon in MAP and vacuum packaging. Potentially by using NanoPack films, the company will be able to increase their exportation value.
- Fresh produce → minimally processed fruits and vegetables (e.g., salads).

Furthermore, the results of the preliminary studies will be used to for rational selection of the food products and the packaging specifications for the pilot studies.

4. Pilot run IV - Industrial Process: three food types packed in bioactive packaging

A product assessment plan will be defined and agreed upon by the food companies that will be demonstrating the use of NanoPack active packaging within their existing food production facilities. This plan will address both the preliminary studies and pilot runs (Pilot IV).

Key indicators of success, benchmarks for comparison and parameters to measure benefits of packaging will be defined. For example, organoleptic profile of food products, days on 'shelf' with limited microbial growth, and effects on specific natural microbiota of the food product.

The migration of the essential oils and other constituents from the packaging into the packaged food matrix during the shelf life period will be determined by Fraunhofer IVV (WP 6). These tests will be conducted at various time intervals during the food shelf life and compliance with European food-contact regulations will be assessed. Furthermore, the depth of EO penetration in solid foods will be examined. Simultaneously, the microbial growth and the effect on sensory properties during storage in the NanoPack packaging will be tested. The correlation of EO migration with food quality and microbial development will elucidate the anti-microbial effect.

The conditions under which NanoPack films display an optimal microbial reduction will be determined. All the results of the pilot studies will be combined with results of WP6 – toxicity, safety and compliance with EU standards/regulation, as well as, with WP7- LCA, cost benefit analysis and social life cycle. Jointly, these assessments are expected to provide a good indication of commercial potential.

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ANNEX 1.

1. Protocol for testing antibacterial activity of plastic by inhibition of *L. innocua* development in liquid medium

Materials

- **Reference strain** - Gram positive bacteria - *Listeria innocua* ATCC 33090
- **Growth medium -** Brain heart infusion (BHI) media (Difco, cat No. 237500).
  For the preparation of agar plates (BHI agar), bacto-agar (15 g/L) is added to the BHI medium.

Procedure

1. Incubate *L. innocua* (from a -80°C stock) in 3 ml BHI medium at 37°C under agitation of 250 rpm for 16-20 hr (starter).
2. Dilute the starter culture using BH to obtain 3 ml culture at 0.1 O.D.
3. Incubate at 37°C under agitation (250 rpm) until the culture reaches O.D 0.3 (logarithmic culture; 10^8 CFU/ml).
4. Dilute the culture to 1:100 in NB.
5. Add the tested film (disc of **1.2 cm** diameter) into wells (in a 24 well plate) containing 1 ml culture in diluted NB. The plate contains reference wells as followed: NB 1:100 medium, NB 1:100 medium + *L. innocua*.
6. Incubate for 22-24 hr at 37°C under agitation (100 rpm).
7. Transfer 200 µl from each of the 24 wells (samples) into the first column of 96 wells plate
8. Perform a 7 tenfold (10^{-7}) serial dilution for each sample by transferring 20 µl from each well into 180 µl diluted (1:100) NB in the 96 wells plate.
9. Drop test – transferring 10 µl from each sample (including the non-diluted samples at first line) in duplicates onto BHI agar plate and incubate over night at 37°C.
10. Counting bacterial colonies in the drops (*L. innocua* in NB 1:100 medium without film should give 10^{6-7} CFU/ml).

Counting bacterial colonies in the drops. The obtained reduction levels will be designated as follows:

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Reduction	Designation of reduction level	Meaning of designation
$10^{6/7}$ in all experiments	VH	Very high
10^{6}-10^{7}	H	High
10^{3}-10^{5}	M	Medium
10^{1}-10^{2}	L	Low
10^{0}	No	No reduction

2. Protocol for testing antibacterial activity of plastic by inhibition of *E. coli* development in liquid medium

Materials

- **Reference strain** - Gram negative bacteria - *E.coli* ATCC 8739 (based on ISO 22196)
- **Growth medium for *E. coli* -** NB (Fluka; cat No. 70149).
  For the preparation of agar plates (NA), bacto-agar (Becton Dickinson 15 g/L) is added to the NB medium.

Procedure

1. Dilute the starter culture using NB to obtain 3 ml culture in 0.1 O.D.
2. Incubate at 37°C under agitation (250 rpm) until the culture reaches O.D 0.6 (logarithmic culture = 10^{8} cfu/ml).
3. Dilute the culture with NB (1:100) to obtain 10^{4} cfu/ml culture.
4. Add the film saple to be tested (disc of **1.2 cm** diameter) into wells (in a 24 well plate) containing 1 ml culture in 1:100 diluted NB.
5. As a reference, bacterial count of NB 1:100/NB 1:300 medium + *E. coli* is performed (as described below at 6-9) giving 10^{4} cfu/ml.
6. Incubate for 22-24 hr at 37°C under agitation (100 rpm).
7. Transfer 200 µl from each of the 24 wells (sample) into wells of the first column of 96 wells plate
8. Perform a 6 ten-fold (10^{-6}) dilution for each sample by transferring 20 µl from each well into 180 µl 1:100 NB in the 96 well plate.

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9. Drop test – transfer 10 µl from each sample (including the non-diluted samples at first column) in duplicates onto NB plate and incubate over night at 37°C.

Counting bacterial colonies in the drops. The obtained reduction levels will be designated as follows:

Reduction	Designation of reduction level	Meaning of designation
10^7 in all experiments	VH	Very high
10^6-10^7	H	High
10^3-10^5	M	Medium
10^1-10^2	L	Low
10^0	No	No reduction

3. Protocol for testing antibacterial activity of plastic by inhibition of bacterial development by micro-atmosphere on agar plates

Materials

- Reference strain - Gram negative bacteria _ *E.coli* ATCC 8739 (based on ISO 22196)
- **Growth medium** for *E. coli* - NB (Fluka; cat No. 70149).
  For the preparation of agar plates (Nutrient Agar, NA), bacto-agar (Becton Dickinson 15 g/L) is added to the Nutrient Broth (NB) medium.

- Reference strain - Gram positive bacteria _ *Listeria innocua* ATCC 33090
- **Growth medium -** Brain heart infusion (BHI) media (Difco, cat No. 237500).
  For the preparation of agar plates (BHI agar), bacto-agar (15 g/L) is added to the BHI medium.

Procedure

1. Culture bacteria on relevant nutritional agar plates.
2. Incubate at 37°C O.N (overnight; 18h).
3. Transfer one colony of bacteria from plate to 5 ml of relevant nutritional medium
4. Incubate over night at 37°C + agitation at 150 rpm.
5. Spread 100 µl of the culture (bacterial cells at stationary phase – overnight culture, 3.7×10^8 cfu/ml) in selected dilutions or as is on appropriate agar plates.
6. The suggested concentration is 3.7×10^6 cfu/ml that is 1:100 dilution of the overnight culture in Saline solution.

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7. Attach the tested film disc (6-7 cm in diameter) to the lid of the Petri dish. Test film vs. control film in 3 replications each.
8. Incubate for 18h (Overnight) at 37°C.
9. Observe clear zone under the investigated polymer films and compare it to reference films

4. Protocol for testing antibacterial activity of plastic by inhibition of fungi by micro-atmosphere on agar plates

Materials

- Reference microorganism - *Penicillium spp*
- **Growth medium:** Potato Dextrose Agar (PDA, Difco cat No. 213400).

Procedure

1. Grow spores of *Penicillium spp.* on PDA plates in the dark at 25 °C for one week.
2. Harvest the spores and suspend in 5 mL of 0.85% (w/v) saline solution to obtain a conidial spore suspension (CSS) at a concentration of 10^6 conidia / ml.
3. Attach the tested film disc (6-7 cm in diameter) to the center of the Petri dish lid.
4. Needle-inoculate (~20 µl) the PDA (1%) with the CSS at center of the Petri dish.
5. Close the Petri dishes and seal tightly with Parafilm™.
6. Incubate in inverted position for 7 days at 25 °C in the dark.
7. Monitor the radial growth perpendicularly every 2 days, by measuring the colony diameter.
8. Plot the colony diameter on time and calculate the linear growth rate (Kr; linear regression slop).
9. Calculate % growth inhibition to control using the equation: {(Kr control – Kr sample) / Kr control}*100.
10. All tests should be conducted in triplicates.

5. Protocol for testing antibacterial activity of plastic by inhibition of *L. innocua* development by micro-atmosphere method in liquid medium

Materials

- **Reference strain** - Gram positive bacteria - *Listeria innocua* ATCC 33090

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- **Growth medium -** Brain heart infusion media (Difco, cat No. 237500).

For the preparation of agar plates (BHI agar), bacto-agar (15 gr/lit) is added to the BHI medium.

Procedure

1. Incubation of *L. innocua* (from a -80°C stock) in 3 ml BHI medium at 37°C under agitation of 250 rpm for 16-20 hr (starter).
2. Dilution of the starter culture using BHI medium to obtain a 3 ml culture of 0.1 O.D.
3. Incubation at 37°C under agitation of 250 rpm until the culture reaches O.D 0.6 (logarithmic culture).
4. Diluting the culture into BHI 1% to obtain a 10^5 cfu/ml culture.
5. Adding the tested film on top of 2 wells (the film is in a size sufficient to cover the area of 2 wells) in a 6-well plate, so there is no direct contact between the film and the bacterial solution. Each well contains 3 ml culture in a diluted BHI. Due to the evaporation of the oil, each plate contains only one type of film while the rest of the wells are being filed with an equivalent volume of water (i.e. 3 ml). Each experiment contains 2 controls as follows: BHI 1% medium, BHI 1% medium + *L. innocua*.
6. Incubation for 22-24 hr at 37°C under agitation (100 rpm).
7. Transferring 200 µl from each of the tested wells (sample) into the wells of the first line in a 96-well plate.
8. Performing a 7 tenfold dilution from each sample by transferring 20 µl from each well into 180 µl BHI 1% in the second line and from the second to the third and so on and so forth.
9. Drop test – transferring 10 µl from each sample (including the undiluted samples at the first line) in duplicates into NA plates and incubating them at 37°C O.N.
10. Counting bacterial colonies developed in the drops.

The obtained reduction levels will be designated as follows:

Reduction	**Designation of reduction level**	**Meaning of designation**
10^7 in all experiments	VH	Very high
10^6-10^7	H	High
10^3-10^5	M	Medium
10^1-10^2	L	Low
10^0	No	No reduction

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6. Protocol for testing antibacterial activity of plastic by inhibition of *E. coli* development by micro-atmosphere method in liquid medium

Materials

- **Reference strain** - Gram negative bacteria - *E.coli* ATCC 8739 (based on ISO 22196)
- **Growth medium for *E. coli* -** NB (Fluka; cat No. 70149)**.**

For the preparation of agar plates (NA), bacto-agar (Becton Dickinson 15 g/lL) is added to the NB medium.

Procedure

1. Incubation of *E. coli* (from a -80°C stock) in 3 ml NB medium at 37°C under agitation of 250 rpm for 16-20 hr (starter).
2. Dilution of the starter culture using NB medium to obtain a 3 ml culture of 0.1 O.D.
3. Incubation at 37°C under agitation of 250 rpm until the culture reaches O.D 0.6 (logarithmic culture).
4. Diluting the culture into NB 1% to obtain a 10^5 cfu/ml culture.
5. Adding the tested film on top of 2 wells (the film is in a size sufficient to cover the area of 2 wells) in a 6-well plate, so there is no direct contact between the film and the bacterial solution. Each well contains 3 ml culture in a diluted NB. Due to the evaporation of the oil, each plate contains only one type of film while the rest of the wells are being filed with an equivalent volume of water (i.e. 3 ml). Each experiment contains 2 controls as follows: NB 1% medium, NB 1% medium + *E. coli*.
6. Incubation for 22-24 hr at 37°C under agitation (100 rpm).
7. Transferring 200 µl from each of the tested wells (sample) into the wells of the first line in a 96-well plate.
8. Performing a 7 tenfold dilution from each sample by transferring 20 µl from each well into 180 µl NB 1% in the second line and from the second to the third and so on and so forth.
9. Drop test – transferring 10 µl from each sample (including the undiluted samples at the first line) in duplicates into NA plates and incubating them at 37°C O.N.
10. Counting bacterial colonies developed in the drops.

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The obtained reduction levels will be designated as follows:

Reduction	Designation of reduction level	Meaning of designation
10^7 in all experiments	VH	Very high
10^6-10^7	H	High
10^3-10^5	M	Medium
10^1-10^2	L	Low
10^0	No	No reduction

7. Protocol for testing antibacterial activity of plastic by inhibition of bacterial development on agar plates (Kirby-Bauer method)

Materials

- **Reference strain** - Gram negative bacteria - *E.coli* ATCC 8739 (based on ISO 22196)
- **Growth medium for *E. coli* -** NB (Fluka; cat No. 70149)**.** For the preparation of agar plates (NA), bacto-agar (Becton Dickinson 15 gr/lit) is added to the NB medium.

- **Reference strain** - Gram positive bacteria - *Listeria innocua* ATCC 33090
- **Growth medium for *L. innocua* -** Brain heart infusion media (Difco, cat No. 237500).

For the preparation of agar plates (BHI agar), bacto-agar (15 gr/lit) is added to the BHI medium.

Procedure

1. Culture bacteria on relevant agar plates.
2. Incubate at 37°C O.N (overnight; 18h).
3. Transfer one colony of bacteria from plate to 5 ml of relevant nutrient medium.
4. Incubate at 37°C O.N, 150 rpm agitation.
5. Spread 100 µl of the culture (bacterial cells at stationary phase – O.N culture, 3.7×10^8 cfu/ml) in selected dilutions or as is on appropriate agar plates.
   The suggested concentration is 3.7×10^6 cfu/ml that is 1:100 dilution of the O.N culture in Saline solution.

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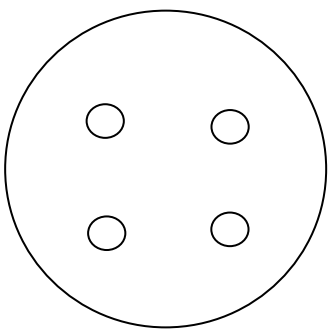
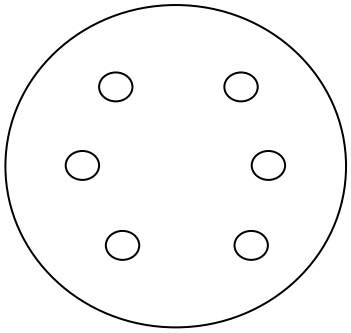
6. Place the tested film discs in diameter of 1.2 -1.5cm on the agar plates, test film vs. control film in 3 replications each. Four or six discs for a plate.





7. Incubate for 18h (O.N) at 37°C.
8. Look for a clear zone (inhibition zone) around the disc films that includes the antibacterial material.
9. Measure the diameter of the clear zone.
10. Calculate the mean of the replications and % inhibition from control.

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