EcoBioCAP

ECOefficient BIOdegradable Composite Advanced Packaging

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Collaborative project

SEVENTH FRAMEWORK PROGRAMME

Priority: Food, Agriculture and Fisheries, Biotechnology

Deliverable D4.2

Report on the microbial stability of some selected packaging materials

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Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium(including the Commission Services)	

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Glossary and definitions

BCNW : bacterial cellulose nanowhiskers
EcoBioCAP: ECOefficientBIOdegradable Composite Advanced Packaging
PEG : Polyethylene glycol
PHA : poly(hydroxy alcanoate)
PHB : poly(3-hydroxybutyrate)
PHBV : poly(3-hydroxybutyrate-co-3-valerate)
PHBV3 :poly(3-hydroxybutyrate-co-3-valerate) with 3% valerate content
PHBV12 : poly(3-hydroxybutyrate-co-3-valerate) with 12% valerate content
RH : relative humidity
WSF : Wheat straw fibers

Summary

Objectives of D4.2. in the framework of WP4

The general objective of WP4 is to investigate the suitability of packaging materials developed in WP3 as food contact materials (FCM). The structural, physical-chemical and microbiological stability of packaging materials under the whole life cycle of packaging materials are the first keys to ensure their ability to preserve food safety within defined limits and recommended usage conditions. Potential physico-chemical and microbial material degradation as well as breakdown products and contaminants migration could occur during the contact of the materials with the food. In this context, the structural and physico-chemical stability of PHBV-based materials with respect to the targeted food packaging application (*i.e.* by studying the influence of severe yet realistic conditions of storage and food contact on the evolution of their mechanical, thermal and barrier properties, in relation to the evolution of their structure at different scales) has been studied in task 4.1. and results were presented in deliverable 4.1. The objective of the present deliverable (D4.2.) is to bring new and necessary insights and understanding into the microbiological stability of some PHBV-based packaging materials by studying the influence of conditions of storage and food contact on the characteristics of bio-films formation in relation to the surface properties of the packaging materials.

Overview of D4.2. main results

The deliverable 4.2 will present the specific methodology used to evaluate the microbial stability of PHBV-based composite and multilayer materials against *Listeria monocytogenes*. *L. monocytogenes* is one of the most relevant foodborne pathogenic bacteria. For the commercial application of this kind of structures, it is important to understand whether these biopolymers favour the growth of pathogenic bacteria. Besides, one of the targeted food product in this project is fresh cheese. In that case*, Listeria monocytogenes* could be a real problem if products are prepared with unpasteurized milk. In this context, it has been decided to evaluate the survival of *Listeria monocytogenes*, as a microorganism model, under different relative humidities condtions. The deliverable 4.2 will include the description of methodologies to detect and monitor the degradation of those materials during ageing. Then, results on the *Listeria* survival at different relative humidities of the selected packaging materials will be presented.
Results show that the relative humidity (RH) and ageing greatly influenced the microbial stability of the studied materials, 100% RH being the worse case. For instance, at 100% RH, high amounts of *Listeria* survived in the samples after 3 months of storage, although in most of the films the growth of yeast and molds interfered in the analyses leading to the difficulty to easily calculate the UFC. It is worth noting that the introduction of 20wt% of wheat straw fibers in PHBV results in a deep decline of *Listeria monocytogenes* titres along the storage time for materials stored at 53% RH. Indeed, in composite materials, *Listeria monocytogenes* only survives until day 15 whereas in virgin PHBV films, *Listeria monocytogenes* is still detected after 4 weeks of storage. This behaviour can be related, on the one hand, with the antimicrobial effect of polyphenols contained in the lignin of wheat straw fibers. On the other hand, the presence of residual physicochemical compounds inside the straw could contribute to the deep decline of *Listera Monocytogenes* titres for materials stored at 53% RH.
Results are of great interest since *L. Monocytogenes* does not survive at low relative humidities, which could provide a safe application for food products stored at low RH.

Teams involved

5 teams are involved in WP4 : INRA, Fraunhofer, UNIROMA, UMINHO and CSIC, with 60.9 P/M for the whole WP (ending at 30 months for T4.1 and at 32, 36 and 42 months for T4.2, T4.3 and T4.4, respectively) (Table 1). 24.5 P/M are devoted for task 4.2., with only CSIC and INRA involved.

Table 1. Repartition of PM per team involved in WP4

Participant number [10]	Participant short name [11]	Person-months per participant
1	INRA	24.00
2	Fraunhofer	5.00
5	UNIROMA	8.00
7	UMINHO	15.00
9	CSIC	8.90
	Total	60.90

Results and discussion

1. Methodology to evaluate the microbial stability of PHA based materials against *Listeria monocytogenes*

1.1. Selected materials

The selected materials used for the microbiological stability of PHBV-based materials are presented in Table 2. Microbial stability has been evaluated on the first generation of materials obtained in task 3.1: nacocomposites based on BNCW or feather fibers and multilayers systems based in PHBV3 and PHB-zein. Multilayer structures have been chosen as good candidates to be scaled-up. Furthermore, since PHBV3/wheat straw fibers composite materials have been chosen for the up-scaling of the preparation of trays (WP6), materials prepared by INRA were also analyzed by the CSIC parther as regards their microbiological stability. The protocols of preparation are detailed in the following paragraphs.

Table 2. Overview of materials used in Deliverable 4.2.

Nanocomposites *Prepared by CSIC* • PHBV3 (Tianan) / BCNW (3wt%) • PHBV12 (Goodfellow) / feather fibers (10 and 50wt%) *Prepared by INRA* PHBV3 (Tianan) / Wheat straw fibers (20wt%)
Multilyers *Prepared by CSIC* • PHBV3-PEG / Zein / PHBV3-PEG • PHB (Biomer) / Zein / PHB

Nanocomposites films.

Two PHBV matrices have been chosen for the preparation of nanocomposite films by CSIC. On the one hand, PHBV produced by Tianan (with 3% of valerate, notes PHBV3) was chosen since it seems to be a good candidate for large-scale production due to its relatively easy processability. 3 wt% of bacterial cellulose nanowhiskers (BCNW) were incorporated into PHBV3 by melt

compounding. On the other hand, 10 and 50 wt% of feather fibers were introduced in PHBV containing 12% of valerate (noted PHBV12) (Goodfellow Cambridge Limited, Huntingdon, England) (Martínez-Sanz, et al., 2013, Pardo et al., 2013).

Multilayers films.
Multilayer structures were prepared by CSIC with plasticized PHBV3 (with Polyethyleneglycol 900 (PEG) Sigma–Aldrich, Madrid, Spain) and non-plasticized PHB provided by Tianan and Biomer, respectively. To this end, once electrospun hydrocolloid zein interlayer was collected onto PHBV3-PEG or PHB films, they were covered with another similarly prepared film. The resulting system was compressed in a hot press at 160°C for 1 min.

Composite films.
Composite films based on PHBV3 (from Tianan) and 20 wt% of impact milled wheat straw fibers (IM-WSF) obtained by INRA as described in details in the deliverable 3.1. of EcoBioCAP project (noted PHBV/20%WSF in the following deliverable). Briefly, biocomposites were compounded by extrusion using a lab-scale twin-screw extruderwith a L/D = 40 and a screw diameter of 16mm (Eurolab from ThermoFisher Scientific).The temperature profile from the polymer feeding to the die varied from 180°C to 160°C. After extrusion, the obtained dry compounds were heated 5 min at 170°C between two Teflon-coated plates and then thermo-molded for 5 min at 150 bar and 170°C with a heated hydraulic press (PLM 10 T, Techmo, Nazelles, France) to obtain films (about 250 µm thick) for further characterization. A wheat strawfiber content of 20wt% was used, using the medium fraction obtained by impact-milling displaying sizes of 100-150µm (the whole processing and characterization protocols of wheat straw fibers are presented in Deliverable 2.1 of EcoBioCAP project.

1.2 Simulation of storage conditions

Effect of relative humidity. The effect of relative humifity was evaluated by CSIC. Samples were equilibrated in desiccators at four different relative humidities (0, 53, 75 and 100%) at 25°C by using, respectively, silicagel, oversaturated solutions of $Mg(NO_3)_2$, NaCl and distillated water for three months in order to perform the analysis. The microbiology was analyzed at different times (0, 15, 30, 45, 60 and 90 days). The testing period (three months) was chosen to cover the lifetime of the food products selected in the EcoBioCAP project.

1.3. Evaluation of the microbial activity of PHA-based biocomposites against *L. monocytogenes* : Which criteria and which methods?

L. monocytogenes is one of the most relevant foodborne pathogenic bacteria and, for commercial applications of these biopolymers, it is important to know the behaviour of them against the *Listeria* growth. Furthermore, *L. monocytogenes* is a pathogen microorganim usually found in fresh-cheese (targeted product in this project), which has been prepared with unpasteurized milk. Thus, the microbiological stability of selected packaging materials developed by each partner in WP3 will be evaluated by studying the microbiological activity against *Listeria monocytogenes*. Microbiological test of all materials were carried out by CSIC. The microbiological stability of the selected materials were determined by adapting the methology used by Kristo, Koutsoumanis and Biliaderis (2008). It is worth to note that this type of microbiological tests are usually used for biopolymers containing antimicrobial agents but not in this kind of polymers.

1.3.1 Bacterial strains and preparation of inoculum

Listeria monocytogenes CECT 5672 was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain). This strain was stored in Phosphate Buffer Saline
(PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories,Madrid, Spain) and 10% glycerol at -80°C until needed. For experimental use, the stock culture was maintained by monthly subculturing to agar Tryptone Soy Agar (TSA) slants at 4°C. Previous to each study, a loopful of bacteria was transferred to 10 ml of TSB and incubated at 37°C overnight. A 100 µl aliquot from the overnight culture was again transferred to TSB and grown at 37°C to the mid-exponential phase of growth. This culture served as the inoculum for the assays.

1.3.2 Inoculation of films

A *L. monocytogenes* suspension so as to achieve 5 × 10^5 CFU/cm2 was applied onto the test films of 1×1 cm. Samples were stored for three months in desiccators at four different relative humidities (0, 53, 75 and 100%) at 25ºC as described above. The studied films (Table 2) were taken from the desiccators at different time intervals (0, 7, 15, 28, 42, 60 and 90 days) and bacteria were then recovered and were 10-fold serially diluted in 0.1% buffered peptone water (BPW) and plated on TSA for plate counts after incubation at 37°C for 24 h. Each experimental condition was analyzed in duplicate. Moreover, a different set of films without bacteria inoculum were stored at the above mentioned conditions in order to evaluate the growth of yeast and molds by visual inspection.

1.4. Contact angle measurements

Surface hydrophobicity of PHBV-based composite films (PHBV3 filled with 20wt% of WSF) were evaluated at room temperature by contact angle measurements using a contact angle meter (Digidrop, GBX, France) equipped with a diffuse light source and a CCD camera (25 frames per second). The dispersive (γ_S^d) and polar (γ_S^p) components of the solid surface tension were evaluated by applying the Owens-Wendt approach (Owens and Wendt, 1969). For this purpose, five reference liquids were used: formamide (Acros organics, Geel, Belgium), diiodomethane (Acros organics, Geel, Belgium), ethylene glycol (Aldrich chemical Co. Inc., Milwaukee, USA) and glycerol (Merk, Darmstadt, Germany). Their respective surface tension components were taken from the literature and are listed in Table 3 (Angellier et al., 2005; Michalsky et al., 1998; Owens and Wendt, 1969; Van Oss, 1994).

Table 3. Surface tension components (γ_L^d, dispersive component; γ_L^p, polar component and γ_L, total surface tension) of the reference liquids.

Liquids	γ_L^d (mJ.m^{-2})	γ_L^p (mJ.m^{-2})	γ_L (mJ.m^{-2})	Reference
Water	21.8	51.0	72.8	(Angellier et al., 2005)
Formamide	39.0	19.0	58.0	(Owens and Wendt, 1969)
Diiodomethane	50.8	0.0	50.8	(Michalsky et al., 1998)
Ethylengycol	29.0	19.0	48.0	(Van Oss, 1994)
Glycerol	34.0	30.0	64.0	(Van Oss, 1994)

2. Microbial stability of selected materials upon storage conditions against *Listeria monocytogenes* : influence of relative humidity.

2.1 Microbial stability of nanocomposites and multilayer films

L. monocytogenes is one of the most relevant foodborne pathogenic bacteria. For the commercial application of this kind of structures, it is important to understand whether these biopolymers favour the growth of pathogenic bacteria. It is worth to highlighted that this kind of microbiological analyses are usually used for biopolymers with antimicrobial agents. However, from the best of our knowledge, there is no information about the effect of these structures in the growth of listeria. Therefore, the effect of relative humidity and ageing on microbiology parameters was evaluated. Figures 1-4 show that relative humidity greatly affected the survival of *Listeria* in multilayer structures and nanocomposites with BCNW or feather fibers. At 100%, high amounts of *Listeria* survived along the storage time, however in most of the films the growth of yeast and molds interfered in the analyses (Figures 1, 2, 3 and 4). It may be noted that the incorporation of zein into the multilayer structures (Figure 1 and 2), did not modified the behaviour of *Listeria* survival. Furthermore, *Listeria* survival at 0%RH of PHBV12 was significanly higher in the case of the addition of feather fibers (Fig 4).

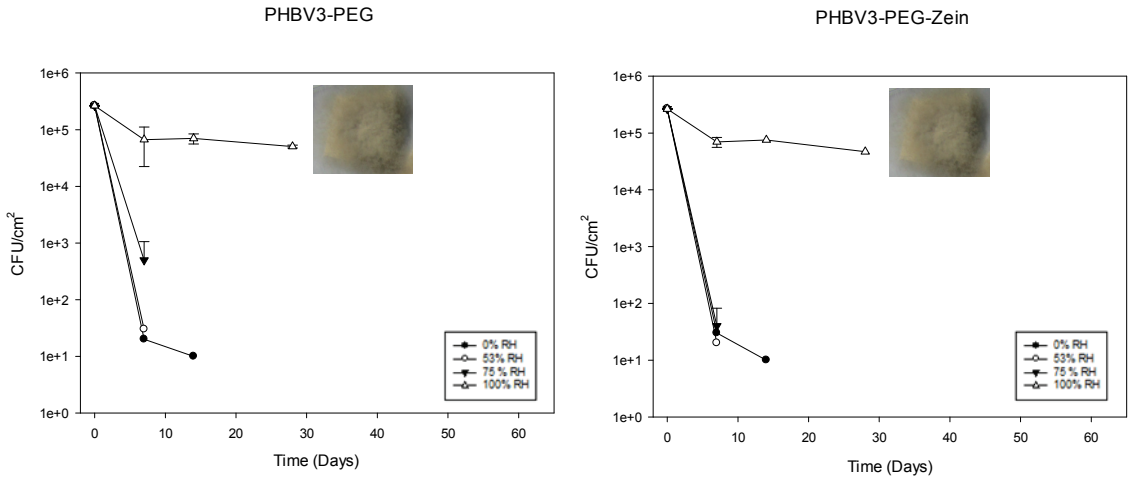

Figure 1 : Survival of *Listeria monocytogenes* inoculated into multilayer materials based on PHBV3-PEG and either with or without a zein interlayer along the storage time and presence of yeast and molds, at four relative humidities. : 0%RH (black circle), 53%RH (white circle), 75 (black triangle) and 100 %RH (white triangle).

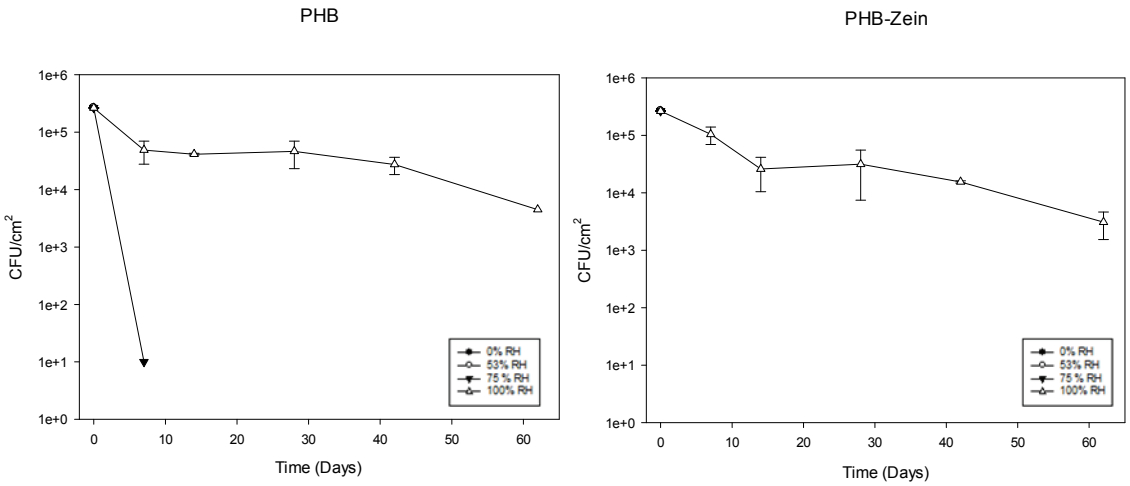

Figure 2 : Survival of *Listeria monocytogenes* inoculated into multilayer PHB with and without zein interlayer along the storage time at 75 (black triangle) and 100 %RH (white triangle).

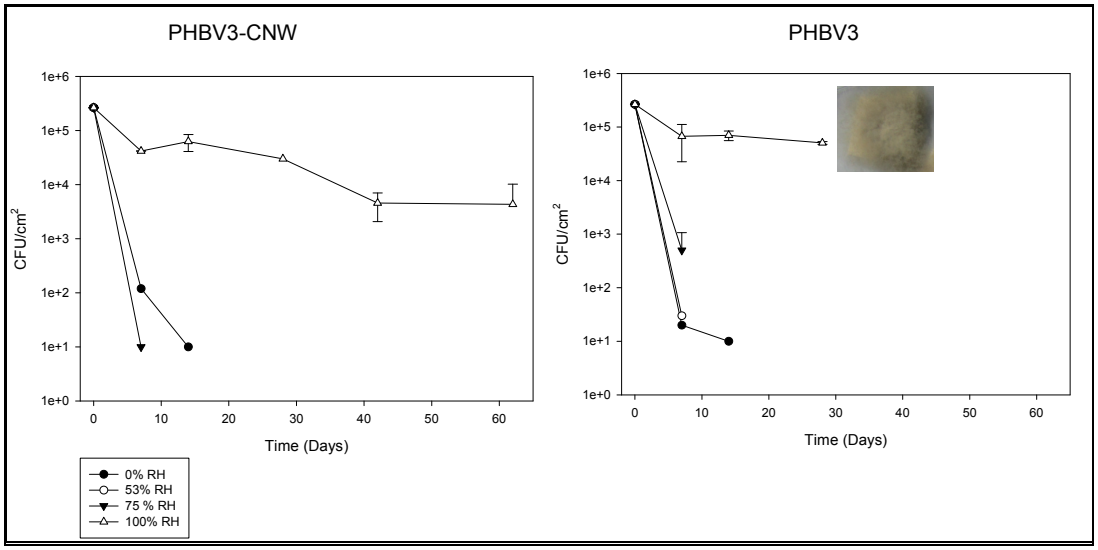

Figure 3 : Survival of *Listeria monocytogenes* inoculated into nanocomposites based on PHBV3 with bacterial cellulose nanowhiskers along the storage time and presence of yeast and molds at four relative humidities : 0%RH (black circle), 53%RH (white circle), 75 (black triangle) and 100 %RH (white triangle).

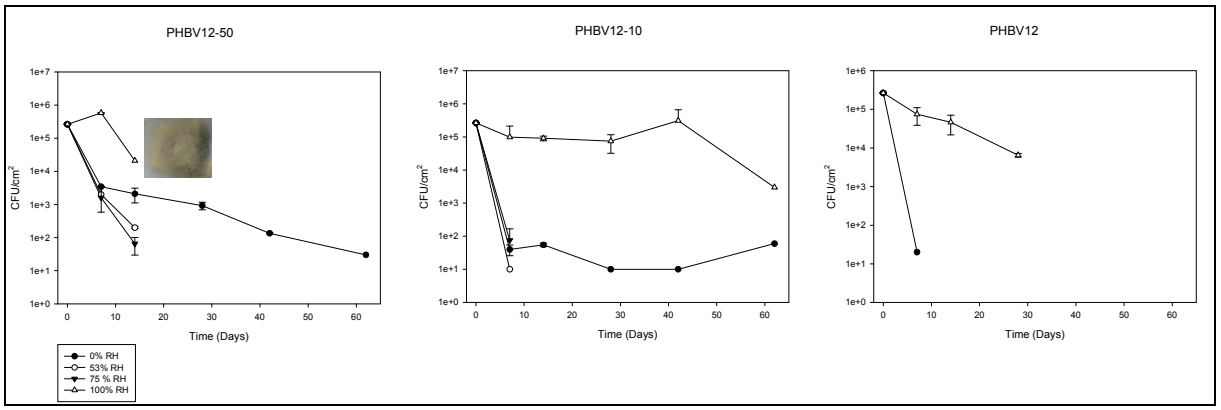

Figure 4 : Survival of *Listeria monocytogenes* inoculated into composites based on PHBV12 with 10 or 50% of feather fibers along the storage time.

2.2 Microbial stability of composite films

The effect of relative humidity and ageing on microbiology parameters of PHBV3 and PHBV3 containing 20% wheat straw fibers was also evaluated by CSIC. As shown in Figure 5, the relative humidity affected the survival of *Listeria*. At 100%, high amounts of *Listeria* survived along the storage time, however in films containing 20% wheat, the growth of yeast and molds interfered in the analyses. On PHBV containing 20% wheat straw fibers kept at 53% RH, *Listeria* titres deeply declined along the storage time, surviving only until day 15. While in PHBV films stored at 53% RH, *Listeria* was detected after 4 weeks of storage. This behaviour can be related with the antimicrobial effect of physico-chemical compounds such as polyphenols contained in wheat straw (Dong et al., 2011; Plumed Ferrer et al., 2013). Listeria survival at 0 and 75 %RH was similar in both films.

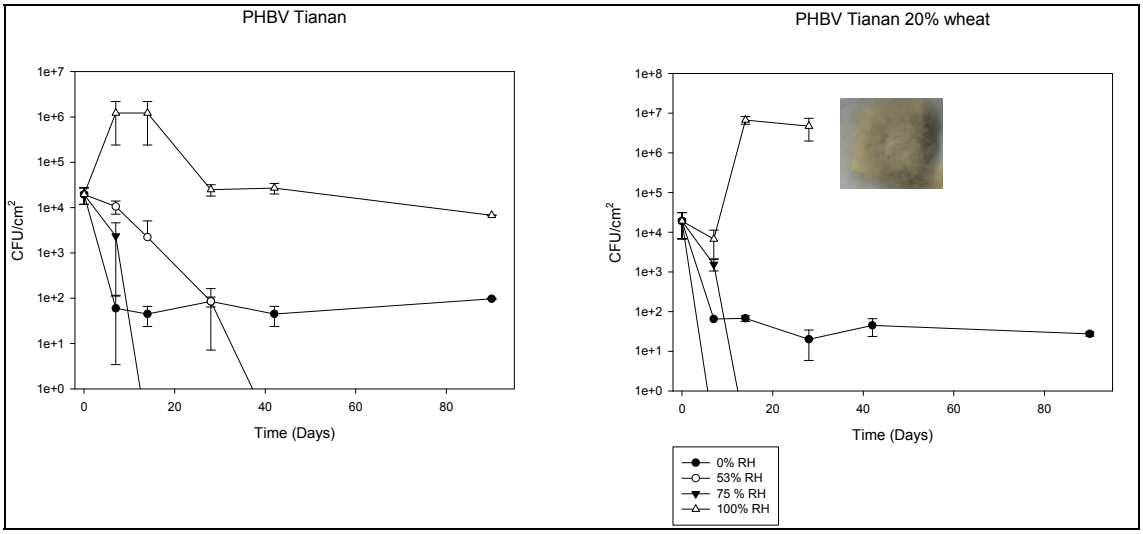

Figure 5 : Survival of *Listeria monocytogenes* inoculated into composites based on PHBV3 and PHBV3 containing 20% wheat straw fibers along the storage time and presence of yeast and molds.

Table 4 shows contact angle measurements of films with and without wheat straw fibers. It could be expected that microorganims would survive more easily on the surface of more hydrophilic films. However, contact angle measurements showed that no significant difference in surface energy was induced by the presence of wheat straw fibers.

Table 4. Contact angle measurements and surface energy of PHBV3 films with and without wheat straw fibers.

Formulation	Contact angle (°)				Surface energy (mJ/m2)		
	Water	Diiodomethane	Glycerol	Ethylene-Glycol	Total surface energy	Dispersive component	Polar component
PHBV	84,7 ± 3,1	42,9 ± 3,2	90,2 ± 7,9	75,3 ± 2,9	30.2	28.3	1.9
PHBV-20%WSF	86,5 ± 4,8	42,9 ± 3,2	100,0 ± 4,1	74,3 ± 3,0	28.5	27.3	1.2

Conclusions and perspectives

It was demonstrated that the relative humidity (from 0 to 100% RH) greatly influenced the *Listeria* survival. Concerning to multilayer structures, only storing at 100% RH, allowed *Listeria* survival after three months of storage, which was not influenced by the presence of a zein interlayer. The effect of relative humidity was similar in nanocomposites prepared by CSIC and composites prepared by INRA, showing that at 100% RH, high amounts of *Listeria* survived along the storage time althougth in most of the films the growth of yeast and molds interfered in the analyses. It is worth noting that the introduction of 20 wt% of wheat straw fibers in PHBV results in a deep decline of *Listeria Monocytogenes* titres along the storage time for materials stored at 53% RH. Indeed, in composite materials, *Listeria Monocytogenes* only survives until day 15 whereas in virgin PHBV films, *Listeria monocytogenes* is still detected after 4 weeks of storage. This behaviour can be related, on the one hand, with the antimicrobial effect of polyphenols contained in the lignin of wheat straw fibers. On the other hand, the presence of residual prytochemical compounds inside the straw could also contribute to the sharp decrease in the listeria growth.

So, these results are of great interest since Listeria monocytogenes does not survive at low relative humidities, which could provide a safety application in foods stored at low RH. However, applications at high relative humidities could be compromised in the case of virgin PHBV based films.

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