Optical, mechanical, and antimicrobial properties of bio-based composites of poly(L-lactic acid) and D-limonene/β-cyclodextrin inclusion complex

Monika Dobrzyńska-Mizera[1], Monika Knitter[1], Daria Szymanowska[2], Salvatore Mallardo[3], Gabriella Santagata[3], Maria Laura Di Lorenzo[3]*

M. Dobrzyńska-Mizera, Institute of Materials Technology, Polymer Division, Poznan University of Technology, 60-965 Poznan, Poland
E-mail: monika.dobrzynska-mizera@put.poznan.pl

M. Knitter, Institute of Materials Technology, Polymer Division, Poznan University of Technology, 60-965 Poznan, Poland

D. Szymanowska, Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 60-627 Poznan, Poland

S. Mallardo, National Research Council (CNR), Institute of Polymers, Composites and Biomaterials (IPCB), c/o Comprensorio Olivetti, via Campi Flegrei, 34, Pozzuoli, NA 80078, Italy

G. Santagata, National Research Council (CNR), Institute of Polymers, Composites and Biomaterials (IPCB), c/o Comprensorio Olivetti, via Campi Flegrei, 34, Pozzuoli, NA 80078, Italy

M. L. Di Lorenzo, National Research Council (CNR), Institute of Polymers, Composites and Biomaterials (IPCB), c/o Comprensorio Olivetti, via Campi Flegrei, 34, Pozzuoli, NA 80078, Italy

Keywords: Bio-based composites, antibacterial properties, PLLA, β-cyclodextrin, D-limonene

Composites made of poly(L-lactic acid) (PLLA) and β-cyclodextrin / D-limonene inclusion complex (CD-Lim) are prepared to develop novel food packaging material with antibacterial properties. The composites are formulated with bio-based materials that are also biodegradable. The addition of CD-Lim to PLLA results in enhanced permeability and water uptake. Optical properties of PLLA/CD-Lim composites also significantly vary compared to plain PLLA, with partial loss of transparency and gloss, but sizably increased barrier to UV light, which imparts protection from oxidation to lipid-containing food. The mechanical properties of the composite films are also affected by composition. Most notably, PLLA films containing CD-Lim display significant antibacterial and antifungal properties, proving their potential as active food packaging films.

1. Introduction

Active food packaging based on bio-based and biodegradable polymers like poly(L-lactic acid) (PLLA) is gaining ground nowadays, as it can prolong food shelf life and maintain its nutritional and visual qualities.[1] PLLA is currently used for food wrapping thanks to its balanced mechanical, optical, and permeation properties, but less developed in the active food packaging field.[2]

Active packaging improves or maintains food quality by controlling temperature and gases in the headspace or limiting bacteria growth, thus extending food shelf life. These packaging types may include temperature sensors or additives able to tailor headspace composition, like gas and moisture absorbers or CO_2/ethanol releasing systems.[2] Of particular interest is antimicrobial food packaging, i.e., a type of packaging that contains additives able to kill pathogens or inhibit their growth.[3,4] Currently, essential oils are the most frequently chosen for this role.[5–9]

In recent years, vast literature data deal with antimicrobial activity of essential oils incorporated into biodegradable polymers, mainly PLLA.[1,10,11] D-limonene (Lim) is a natural antimicrobial agent[12–14] approved by the Federal Register as a harmless flavoring agent and food preservative.[15] Its antibacterial activity was proven with various food-related pathogens, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella enterica*, *Saccharomyces bayanus*, etc.[16,17] However, incorporating D-limonene into a plastic packaging film is not straightforward since its boiling point is well below most polymers' processing temperature: this generates a significant technological barrier leading to its partial or complete evaporation upon material processing. Evaporation of D-limonene upon melt mixing with PLLA was quantified by Arrieta et al.,[18,19] who reported pronounced evaporation of the essential oil during processing. Moreover, high-temperature processing leads to a loss of antimicrobial activity.[20] To make matters worse, the liquid state of D-limonene at room temperature pushes researchers to use different laboratory mixing methods such as solvent cast technique, surface modification, or batch mixers,[1,18–20] which do not allow for large-volume production, hence lack of interest for industrial production.

One of the methods to overcome these problems is encapsulation of D-limonene inside cyclodextrin (CD) cells, which was proven to significantly increase thermal stability of the essential oil.[21] Cyclodextrins molecules create inclusion compounds with different non-ionic, aliphatic, and aromatic substances. They can be utilized to incorporate antimicrobial molecules, whose controlled release may elevate the quality of the wrapped food article and lengthen its

lifetime.[21,22] Worth noting is that encapsulation does not limit the antibacterial or antifungal properties of the entrapped molecules.[11] This is a good prognosis for the antimicrobial inclusion complexes whose enhanced thermal stability and modified state can ease polymer processing, including industrially used processing methods of packaging materials such as injection molding or extrusion.

In our previous manuscript, we detailed the encapsulation of D-limonene within cyclodextrin cavities and introduction of CD-Lim inclusion complex into PLLA *via* melt extrusion to prepare composites with various filler content.[23] We proved with careful design of processing parameters, that the encapsulation within cyclodextrin is a successful way to raise thermal stability of the essential oil. Despite small amount of D-limonene (~ 1.5 wt%) was released from CD-Lim upon melt processing, resulting in Lim dissolved within PLLA amorphous chain portions, still sizable fraction of D-limonene remained trapped within β-CD cavities.[23] A slight plasticization of the polymer was noted, resulting in anticipated onset of cold crystallization in the composites, compared to plain PLLA. Nevertheless, the plasticizing effect was overwhelmed, in terms of mechanical properties, by a presence of high content of *β*-cyclodextrin particles inside the polymeric matrix since microscopic images evidenced quite homogeneous filler dispersion, with some particle agglomeration noted at the highest CD-Lim content (30 wt%).

The initial material characterization detailed in [23] is completed by analyzing the films' tensile behavior and determining the barrier and optical properties of PLLA/CD-Lim, as presented in this manuscript. Most importantly, antibacterial properties against the rise of a wide variety of different bacteria, both gram-positive and -negative, as well as fungi, are also discussed to evaluate the relevance of these materials for active nourishment packages.

2. Experimental

2.1. Materials

A poly(lactic acid), LX175, later named PLLA, characterized by MFR 6 g/10 min (210°C, 2.16 kg), was kindly provided by Corbion (Netherlands). The PLLA with a density of 1.24 g/cm^3, stereochemical purity of 96% (L-isomer), melting temperature of 155°C, and glass transition temperature of 60°C was provided in a form of yellowish pellet. Pure *β*-cyclodextrin (≥ 99%), abbreviated *β*-CD, in a form of white, soluble in water (18 g/L at 25°C) powder was supplied by Cyclodextrin Shop (Netherlands). The residue on ignition and heavy metals amount in *β*-

CD, as designed by the producer as per USP standard, were ≤ 0.1% and ≤ 5ppm, respectively. The constituents were dried for 24h under vacuum at 50°C before extrusion. The technical grade of D-limonene with an approx. purity of 90% was provided by Sigma Aldrich. D-limonene boiling temperature and density were 176-177°C and 0.842 g/mL at 20 °C, as specified by the manufacturer.

2.2. Preparation of the inclusion complex and composites

Incorporation of D-limonene within *β*-cyclodextrin cages was conducted through the precipitation method, as detailed in our previous manuscript.[22,23] Firstly, PLLA was blended with inclusion complex in a rotary mixer Retsch GM 200 (3 min at a speed of 2000 rpm). Secondly, the premixed components with different modifier contents (0 – 30 wt %) were homogenized via co-rotating extrusion with a Zamak extruder at 190°C and 60 rpm. The rod-shaped extrudate was chilled in air and cut. The obtained compositions are summarized in **Table 1**.

Table 1. Symbols and mass concentrations of samples

Designation	Mass concentration [wt %]		
	PLLA	CD-Lim	Lim concentration in composites evaluated based on TGA [%]*
PLLA	100	0	0
PLLA/20CD-lim	80	20	1.4
PLLA/30CD-lim	70	30	2.1

* The content of Lim was evaluated based on thermogravimetric analysis as detailed in [23]

The 1 mm thickness samples were obtained via compression molding method with a Collin Laboratory Forming Press P 200 E. In the first 3 min stage of the process, 190°C and no pressure were applied to permit full melting. Afterward, a pressure of 200 bar was used for another 3 min followed by cooling in air to room temperature. The method was reconducted to produce 150 µm films for optical and barrier properties' analysis.

2.3. Methodology

2.3.1. Water Permeability

Water vapor permeability (WVP) was evaluated by a gravimetric method according to the ISO 12572 standard.[24] Circular test samples with a thickness of approx. 150 µm were prepared and tested for 35 days at 23 ± 5 °C and relative humidity of 50 ± 5%. The specific exchange surface

was 0.00317 m^2, and a sample diameter was equal to 63.5 mm. To achieve one directional moisture flow, the specimens were sealed in aluminum cups containing distilled water, providing 100% Rh humidity. The WVP value for the film samples was calculated according to **Equation 1**:

$$WVP\ (g/m \cdot s \cdot Pa) = (\Delta m \times e) / A \times \Delta t \times \Delta P) \quad (1)$$

where: Δm is mass change (g), e is sample thickness (m), A is exposed surface area (m^2), Δt is the exposure time (s), ΔP (equal to 1,404 Pa) is the difference between water pressure inside and outside the cup corresponding to 50 % Rh at 23°C.[25]

2.3.2. Water Absorption

Water absorption was assessed as per PN-EN ISO 62.[24] Samples were vacuumed at 50°C for 24h before immersion in water at 23°C. The tests were conducted for 14 days. Mass changes were registered periodically by removing the specimens from water and weighting on a Radwag XA 52/2X weight with an accuracy of 0.01 mg. The water uptake (M_t) was determined by **Equation 2**:[24]

$$M_t(\%) = \frac{W_w - W_d}{W_d} \times 100\% \quad (2)$$

where: W_d is the weight of dry specimens (prior immersion), W_w is the weight of composites after exposure to water.

2.3.3. Gloss

The measurements were carried out on PLLA-based films using a gloss meter produced by Rhopoint, model Novo-Gloss Lite, at the angle of 20°. The procedure was conducted ten times for each sample to ensure reproducibility.

2.3.4. Haze

Transparency measurements were carried out on the compression molded films as per PN-84/C-89100 standard.[26] The measures, conducted on a Haze Meter HM-150 by Murakami Color Research Laboratory (Japan), were performed ten times for each sample to ensure reproducibility.

2.3.5. Color Analysis

Color characteristics were determined via CIELAB color space system, which may be pictured as a circular coordinate system. The vertical axis, varying from 0% to 100%, is the lightness L^* (hue), and the radii, namely a^* and b^*, correspond to chroma. Variable a^* shifts from green (-a^*) to red (a^*), whereas variable b^* shifts from blue (-b^*) to yellow (b^*).[2,27] The analysis was carried out using a HunterLab Miniscan MS/S-4000S spectrophotometer. The calibration was conducted on a white standard. Each measurement was conducted ten times at random locations over the sample, and as a consequence, mean values were calculated. Total color differences (ΔE) was evaluated as per **Equation 3**.

$$\Delta E = \sqrt{\Delta a^2 + \Delta b^2 + \Delta L^2} \quad (3)$$

ΔE values are the observer's visual impressions, i.e., the observer cannot notice any color change when the ΔE value is within the range 0 and 1; for the value ranging between 1 and 2, merely a knowledgeable observer may perceive a variation; between 2 and 3.5 everyone can notice the color difference; for the ΔE values above 5 the observer see two different colors.[2] The yellowness index (*YI*) was estimated, as per the ASTM E313 norm,[28] to describe the change in color upon CD-Lim addition from clear to yellow.[18] *YI* was determined according to the tristimulus values *X, Z,* and *Y*, according to **Equation 4**.

$$YI = (100(C_X X - C_Z Z))/Y \quad (4)$$

where: *X*, *Z,* and *Y* are CIELAB tristimulus values, and the C_X, equal to 1.2769, and C_Z, equal to 1.0592, are the coefficients per norm.[28]

2.3.5. UV-Vis

Light absorption was analyzed using a UviLine 9400 spectrophotometer in a range between 100 and 1000 nm. Measurements were carried out using a quartz cuvette, and air as the reference.

2.3.6. Mechanical properties

The tensile tests were performed on a static tensile machine with a crosshead speed of 2 mm min^{-1} using an Instron 5564. As per ISO 527-1[29] standard, the following features were assessed: Young's modulus (E) and elongation at break (ε_b). Ten samples were used for each formulation to guarantee duplicability.

2.3.7. Antibacterial properties

Antimicrobial activity of the samples against *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATTC 29212, *Clostridium butyricum* ATTC 860, *Lactobacillus fermentum* ATCC 14932, *Lactococcus lactis* ATCC 11454, *Streptococcus thermophilus* ATCC 19258, *Bifidobacterium bifidum* ATCC 11863, *Bacillus subtilis* ATCC 6633, *Streptococcus thermophilus* ATCC 19258, *Bifidobacterium bifidum* ATCC 11863, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Proteus mirabilis* ATCC 12453, *Klebsiella pneumoniae* ATCC 31488, *Pseudomonas aeruginosa* ATCC 27853, *Alcaligenes faecalis* ATCC 35655, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9776, *Fusarium* spp., *Aspergillus* spp., *Mucor* spp. was estimated in a qualitative manner as per agar diffusion assay. Concisely, 100 microliters bacterial suspension (1,0 x 107 CFU/ml) of the above pathogens was disseminated on the nutrient agar. Limonene, PLLA, and PLLA/CD-Lim composites (6 mm in diameter), priorly sterilized under UV irradiation for 0.5 h, were placed on the plates' surfaces and incubated at 37^{o}C for 24 h. Antibacterial functions were determined by assessing the inhibition zone against the studied pathogens (the more significant inhibition zone, the better material's antibacterial properties). Each sample was tested three times.

3. Results and Discussion

3.1. Optical features of PLLA-based formulations

To assess suitability of PLLA/CD-Lim composites as packaging materials, their optical appearance was evaluated as function of composition.[30] In particular, optical properties like haze, gloss and color were measured, and completed with UV-Vis spectroscopy analysis. The outcomes are shown in **Figure 1, 2 and 3** as well as **Table 2**.

Haze of PLLA/CD-Lim films sizably increases upon addition of the filler to PLLA. As depicted in Figure 1, pure PLLA has a haze value of 20%, which increases, up to nearly 80%, upon CD-Lim addition. In polymer composites, fillers often cause a decrease of film clarity, which is determined by the filler particle size, generally large enough to collide with light and result in a notable haze.[31–33] However, this is not a crucial parameter, since haze values also depend on film thickness and may be easily controlled to design films with the needed optical properties.

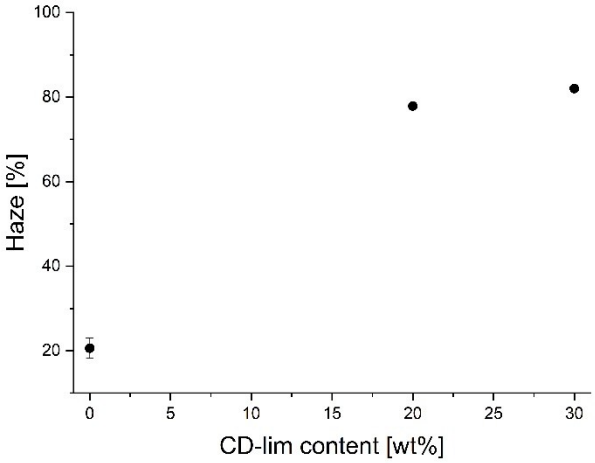

Figure 1. Haze in PLLA/CD-Lim composites as a function of composition

Gloss is also used to assess optical appearance of plastic films and, similarly to haze, in PLLA/CD-Lim composites is affected by composition, as shown in Figure 2. The decrease of gloss with filler addition indicates that the composites have a less brilliant surface, making them less appealing. Again, this does not prevent their usage as food packaging materials. The decreased gloss is coupled with face morphology: the smoother the surface, the glossier the film. Filler addition influences roughness of the films, which results in a decreased reflectance,[34,35] as seen in Figure 2.

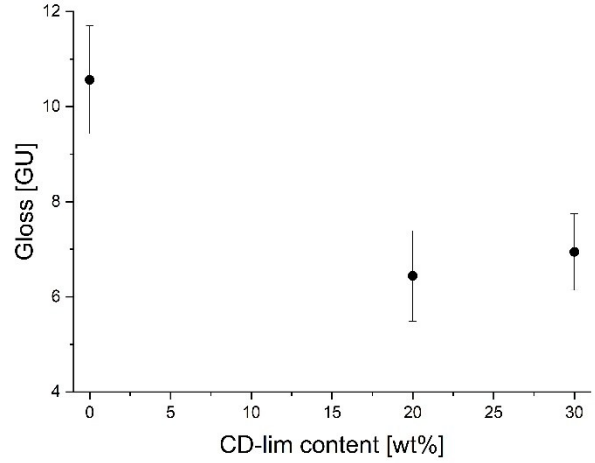

Figure 2. Gloss in PLLA/CD-Lim composites as a function of composition

The absorbance of visible and UV light was measured in the wavelength range of 100 to 1000 nm, with results presented in Figure 3 for the analyzed PLLA/CD-Lim compositions and compared to plain PLLA as well as plain D-limonene. As expected, plain PLLA is highly transparent in the whole visible light wavelength range, with limited absorbance of light only

at wavelength below 300 nm.[36,37] Limonene instead has a sharp absorbance peak in the range between 250 and 350 nm, centered at 293 nm. An absorbance peak is also evident in the PLLA/CD-Lim films, confirming the presence of D-limonene,[22] however slightly shifted to lower wavelengths in the composites due to encapsulation within β-cyclodextrin.[38,39] More importantly, overall transparency of the films is highly reduced in the formulations containing CD-Lim, with a sizable increase in non-transmitted light.

Coupled to the loss in transparency to visible light, caused mainly by whitish cyclodextrin, the composites display also an enhanced absorbance to light in the UV wavelength range. As quantified in Figure 3, nearly all UV light passes through PLLA, whereas incorporation of CD-Lim results in an effective UV blocker absorbing UV light. This finding is of importance for possible exploitation of these films as packaging of lipid-containing food, since UV light promotes the oxidation of lipids, resulting in fast deterioration. Protection from UV light would protect sensitive packaged food from damage, which results in the retention of flavor and visual aspects, shelf-life prolongation, and better product quality.[2] This can be attained upon incorporating CD-Lim within PLLA, unfortunately, coupled with some decrease in limpidity and brilliance of the samples.

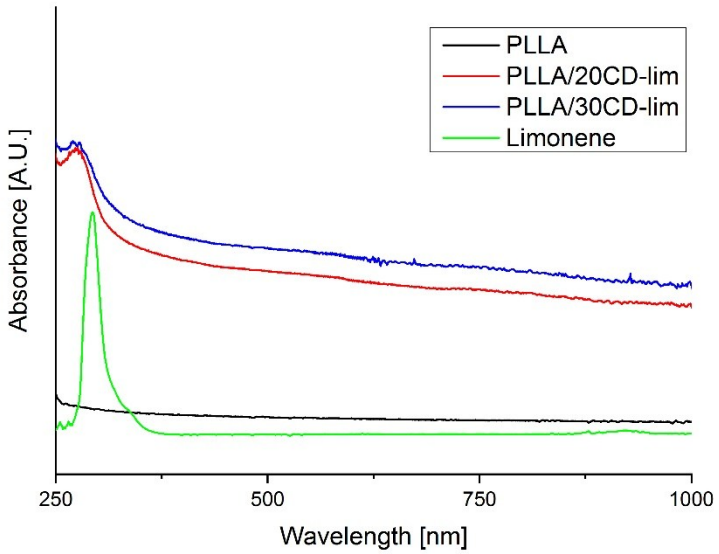

Figure 3. UV-Vis spectra of PLLA/CD-Lim composites

Details of transparency loss are quantified in Table 2, which summarizes the color parameters determined for PLLA and PLLA/CD-Lim films placed on the white background. The lightness value (L^*) decreases slightly upon CD-Lim addition. PLLA/CD-Lim composites still preserve high lightness values, however one should take into consideration that high L^*

values noted for all the tested samples partially were caused by the white background used during the measurement. The *a** parameter is responsible for green (negative) and red (positive) tones. The incorporation of CD-Lim leads to a slight shift towards green tones; however, *a** values in the composites are close to 0, which implies that the minor differences are not perceptible by human eyes. Conversely, *b** markedly vary with sample formulation. In plain PLLA *b** is -6.5 which indicates blue tone in the samples; however, the value moves toward yellow color upon addition of CD-Lim. In order to better estimate those changes, the total color difference (ΔE), according to Equation 3, and the yellowness index, as per Equation 4, were calculated. It was proved that the addition of the filler led to distinct yellowing of the samples, as also evidenced in **Figure 4**, hence the observer would perceive totally different color in case of PLLA/CD-Lim composites, in comparison with pure matrix. A similar tendency in yellowing PLLA upon blending with limonene, was noted in the literature.[18,19] However, at parity of PLLA/Lim ratio, the increase is sizably limited when limonene is encapsulated within β-CD, compared to mere blending.[17]

Table 2. Color characteristics of PLLA/CD-Lim composites

Sample	Color				
	L^*	a^*	b^*	ΔE	YI
White background	93.4 ± 0.1	1.2 ± 0.1	-7.3 ± 0.2	-	-
PLLA	92.5 ± 0.1	1.1 ± 0.1	-6.5 ± 0.2	-	9.3
PLLA/20CD-Lim	86.4 ± 0.6	-0.15 ± 0.1	6.8 ± 0.7	14.7	32.3
PLLA/30CD-Lim	84.5 ± 0.5	0.02 ± 0.1	8.2 ± 0.4	16.7	34.7

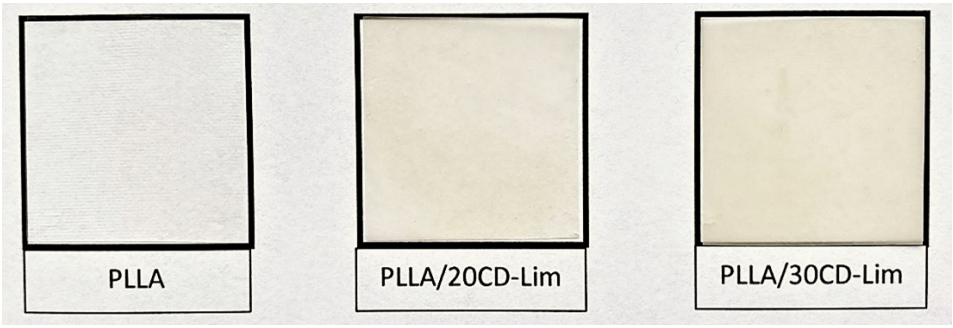

Figure 4. Optical appearance of PLLA, PLLA/20CD-Lim and PLLA/30CD-Lim films

3.2. Barrier properties of PLLA/CD-Lim composites

To evaluate the relevance of films for food wrapping, barrier characteristics were also assessed. Water vapor permeability (WVP) data of PLLA-based formulations are summarized in **Figure 5**. Pure PLLA has a WVP of about $3 \cdot 10^{-11}$ g/m·s·Pa, in line with literature data, with exact data

mainly depending on crystal fraction, crystal polymorphism, and analysis temperature.[2,40] The addition of 20 and 30 wt% of CD-Lim increases water vapor permeability values, which roughly double compared to plain PLLA. The higher permeability to water vapor is likely to be linked to the presence of highly hydrophilic cyclodextrin, whose polar groups can favor water sorption and diffusion into PLLA.[41–44] Slight plasticization of the polyester caused by the partial release of the essential oil [23] also favors the diffusion of water molecules, further contributing to enhanced permeability.

Generally, polylactides are classified as materials with poor water resistance;[45,46] however still can be successfully used for some food applications like selected vegetable, fruits, salads or cheese.[47,48] However, further modification of PLLA/CD-Lim composites needs to be considered to ensure a broader range of application. There are few possible routes to raise water resistance of PLLA, e.g. by increasing crystallinity degree,[40] modifying polymeric matrix with fillers,[46,49] or incorporating PLLA/CD-lim films into multi-layer materials.

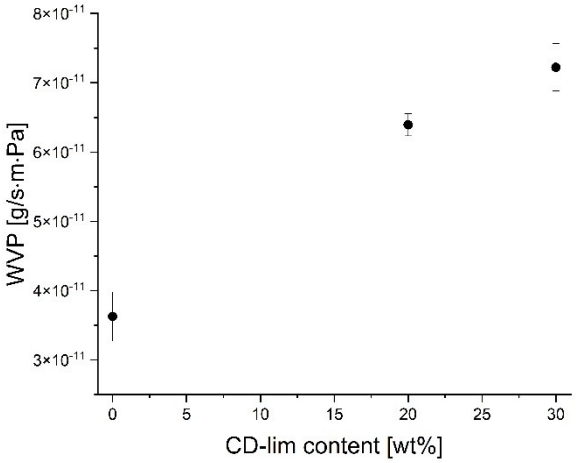

Figure 5. WVP of PLLA/CD-Lim films as a function of CD-Lim content.

The kinetic of moisture absorption in plain PLLA and the composites is compared in **Figure 6**. An instant absorption is noted for all formulations through the initial days of water bathing, much faster in the samples containing CD-Lim. Water uptake levels off at approximately 1% after about 5 days of soaking at 23°C. The CD-Lim complex's addition to the formulation significantly intensifies water absorption in PLLA-based composites together with filler content. Much faster water uptake was monitored in the initial stages of the measurements, with sample saturation attained after about one week, to reach 4.5 and 5.8 wt% in the composites with 20 to 30 wt% filler content, respectively. The above can be related to cyclodextrin's hydrophilic nature linked with the vast availability of hydroxyl groups accessible for interaction with water

molecules.[50] Similar to WVP data, the increase of water uptake is not directly proportional to film composition. This is probably to be linked to partial agglomeration of CD-Lim molecules occurring in formulations with a high amount of filler, probed in [23], which affects not only water absorption kinetics, but also its diffusion through the film, both factors determining the overall permeability to water vapor. The presence of fillers generally results in an increased tortuosity of the diffusion path of vapor molecules, but in the case of particle aggregation, such an increase is limited. On the other hand, the filler is hygroscopic, and higher filler content leads to larger water sorption, as quantified in Figure 5. Balance of the two effects results in WVP that appears independent of film composition for CD-Lim content of at least 20 wt%.

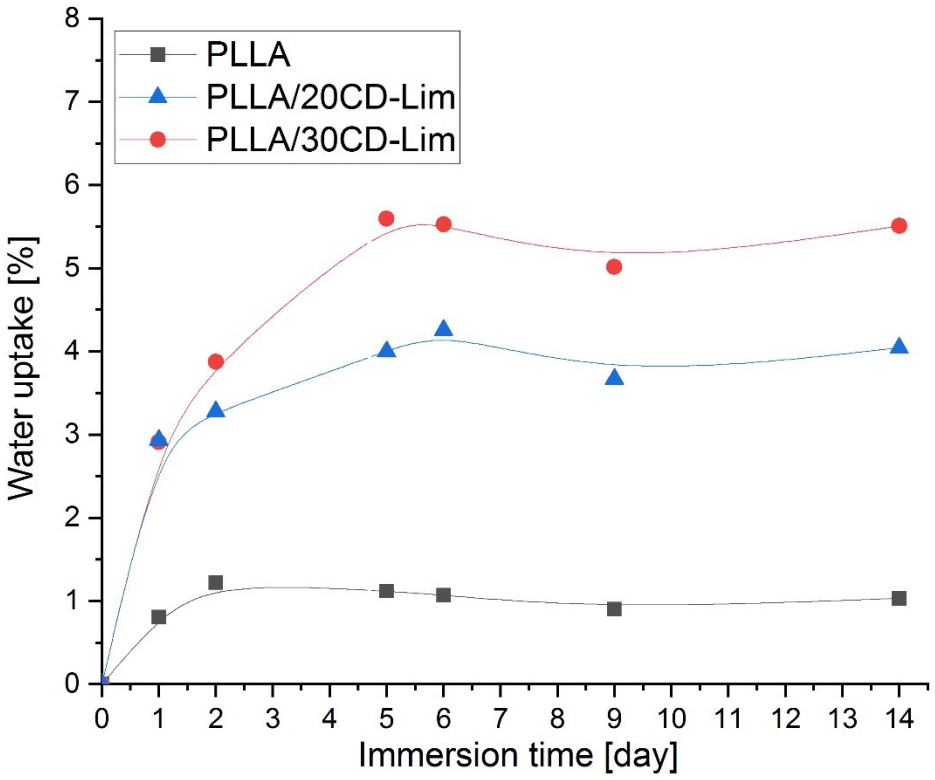

Figure 6. Effect of CD-Lim content on water uptake of PLLA-based composites upon immersion in water at 25°C.

3.3. Mechanical features of PLLA-based formulations

Mechanical properties of PLLA and its composites, including Young's modulus (*E*), tensile strength (*TS*), and elongation at break (ε_b), measured *via* static tensile test, are presented in **Table 3**. Young's modulus value for neat PLLA is 2.1 GPa, in agreement with literature data.[51] The addition of 20 and 30 wt% of CD-Lim into the polymeric matrix does not sizably vary

stiffness of the material, with small changes in measured values that are close to experimental uncertainty, in agreement with DMA analysis detailed in.[23] Instead, elongation at break falls from 5% for neat PLLA down to 1.4% for the PLLA/CD-Lim composites. The decrease in ε_b needs to be rationalized taking into account the composites' morphology, discussed in. [23] CD-Lim particles are homogeneously dispersed within PLLA, but have poor adhesion to the polyester matrix;[23] the latter causes early material failure upon mechanical stress, resulting in increased brittleness. Although pure D-limonene is known[19] for its plasticization effect on PLLA matrix, its activity is limited due to encapsulation in β-cyclodextrin cavities. Our previous research[23] revealed that small amounts of Lim are released from CD-Lim compounds upon heating (e.g. during mixing or shaping in a molten state) and plasticize the PLLA matrix. However, this effect is overwhelmed by a presence of CD-Lim microdomains which determine final mechanical features of the obtained composites. Luckily, this does not exclude the composites as a packaging material, considering that PLLA/CD-Lim composites' tensile strength is not affected by CD-Lim content.[50,52,53]

Table 3. Mechanical properties of PLLA/CD-Lim composites

Sample	Mechanical properties		
	E [GPa]	ε_b *[%]*	*TS [MPa]*
PLLA	2.1 ± 0.3	5 ± 1	26 ± 4
PLLA/20CD-Lim	2.3 ± 0.2	1.4 ± 0.2	30 ± 5
PLLA/30CD-Lim	2.3 ± 0.3	1.3 ± 0.1	26 ± 4

3.4. Antibacterial and antifungal features of PLLA-based formulations

Antimicrobial activity of D-limonene (Lim), poly(L-lactic acid) (PLLA) and PLLA/CD-Lim composites was assessed against 20 indicator strains of a variety of microorganisms, including gram-positive and gram-negative bacteria, and fungi. A unique range of desired and undesirable microorganism species, often found in food, was selected to meet food packaging application requirements. The inhibition zone was measured after spreading every single strain over an agar plate. If the bacteria or fungi are sensitive to the antibacterial agent, an inhibition zone is created. The diameter of the inhibition zone depends on the level of antibacterial or antifungal activity of the formulation, as a more significant inhibition zone implies a more efficient antimicrobial activity.

Inhibition zone data of PLLA/CD-Lim films are presented in **Table 4**, and compared to antimicrobial activity of plain D-limonene and PLLA, tested as references. As expected, PLLA

does not exhibit antibacterial activity to bacteria and fungi. The highest activity was observed for D-limonene against *Listeria monocytogenes* and *Staphylococcus aureus*, both gram positive bacteria, with inhibition zones of 22 and 18 mm, respectively. This result is of high significance for exploitation of PLLA/CD-Lim composites as food packaging material: *Listeria monocytogenes* can cause a severe epidemiological problem, also in the case of products stored under refrigerated conditions[54]. The high efficiency against *Listeria monocytogenes* and *Staphylococcus aureus*, and in general against gram-positive rather than gram-negative bacteria can be linked to the outer membrane layer with lipopolysaccharide molecules of gram-positive microorganisms, which provides a hydrophilic surface.[55] D-limonene is proved to be a potent bacteriostatic agent that destroys bacteria cell wall and membrane, leading to the leakage of intracellular substances, such as nucleic acid and proteins, resulting in cell death. This irreversible damage to the bacteria structure and cell permeability provides an antimicrobial feature by affecting protein expression.[56,57]

In PLLA/CD-Lim composites, D-limonene is partly trapped within β-CD cavities, and part is dissolved within PLLA matrix. As typical for low molar mass molecules, D-limonene diffuses through the polymeric matrix towards surface of the PLLA-based composite,[58-60] whch contributes to enhance antibacterial and antifungal efficiency of the films.

Table 4. Inhibition zones of D-limonene (Lim), poly(L-lactic acid) (PLLA), and PLLA/CD-Lim composites on viable microbial counts.

Microorganisms		Inhibition zone (mm)			
		Lim	PLLA	PLLA/ 20CD-Lim	PLLA/ 30CD-Lim
Gram-positive					
1	*Staphylococcus aureus* ATCC 25923	18.0±2.0	1.0 ±0.0	7.0±1.0	9.0±1.0
2	*Listeria monocytogenes* ATCC 7644	22.0±3.0	0.0±0.0	13.0±2.0	13.0±2.0
3	*Enterococcus faecalis* ATTC 29212	11.0±2.0	0.0±0.0	4.0±0.0	7.0±1.0
4	*Clostridium butyricum* ATTC 860	8.0±1.0	0.0±0.0	2.0±1.0	4.0±0.0
5	*Lactobacillus fermentum* ATCC 14932	9.0±1.0	0.0±0.0	2.0±0.0	3.0±0.0
6	*Lactococcus lactis* ATCC 11454	7.0±1.0	0.0±0.0	2.0±0.0	4.0±0.0
7	*Streptococcus thermophilus* ATCC 19258	9.0±1.0	1.0±0.0	2.0±0.0	4.0±0.0

8	*Bifidobacterium bifidum* ATCC 11863	7.0±1.0	0.0±0.0	1.0±0.0	3.0±0.0
9	*Bacillus subtilis* ATCC 6633	9.0±1.0	0.0±0.0	3.0±0.0	6.0±0.0
Gram-negative					
10	*Escherichia coli* ATCC 25922	5.0±1.0	0.0±0.0	1.0±0.0	2.0±0.0
11	*Salmonella typhimurium* ATCC 14028	3.0±1.0	0.0±0.0	0.0±0.0	1.0±0.0
12	*Proteus mirabilis* ATCC 12453	4.0±1.0	0.0±0.0	0.0±0.0	2.0±0.0
13	*Klebsiella pneumoniae* ATCC 31488	6.0±1.0	0.0±0.0	0.0±0.0	3.0±0.0
14	*Pseudomonas aereuginosa* ATCC 27853	5.0±1.0	0.0±0.0	0.0±0.0	3.0±0.0
15	*Alcaligenes faecalis* ATCC 35655	6.0±1.0	0.0±0.0	0.0±0.0	0.0±0.0
Fungi					
16	*Candida albicans* ATCC 10231	9.0±1.0	0.0±0.0	4.0±0.0	5.0±0.0
17	*Saccharomyces cerevisiae* ATCC 9776	11.0±2.0	0.0±0.0	6.0±0.0	8.0±1.0
18	*Fusarium* spp.	13.0±2.0	0.0±0.0	8.0±1.0	9.0±1.0
19	*Aspergillus* spp.	10.0±2.0	0.0±0.0	3.0±0.0	8.0±1.0
20	*Mucor* spp.	11.0±2.0	0.0±0.0	7.0±0.0	8.0±1.0

The inhibition zone in case of *Staphylococcus aureus* for the film containing 30 wt% CD-Lim is wider than that of PLLA/20CD-Lim composite, namely 9 and 7 mm, respectively. The antimicrobial activity noted for *Listeria monocytogenes* is retained for both compositions showing the inhibition zone of 13 mm. Although, the antibacterial effectiveness is reduced by about one-half for both gram-positive and gram-negative bacteria, in comparison with pure D-limonene, the values are still sufficiently high to provide efficient antibacterial properties for food packaging. It is wort to note that films containing 30 wt% CD-Lim retain most activity against *Saccharomyces cerevisiae*, *Fusarium spp*. and *Aspergillus spp*, with only minor reduction, almost close to experimental uncertainty, in antifungal activity compared to plain D-limonene.

4. Conclusions

Fully bio-based and biodegradable PLLA and CD-Lim composites were prepared *via* melt mixing in various formulations, from 20 to 30 wt% of filler content, and described in the mechanical, barrier, optical, and antibacterial properties. The incorporation of CD-Lim results in enhanced permeability and water uptake due to a presence of hydrophilic filler. Also, CD-Lim added to PLLA leads to reduced gloss and transparency and a more yellowish color. Increased Young's modulus values upon modification were also noted, as expected. Food packaging films should maintain optimal barrier properties and high transparency as per consumer needs. Worth noting is that their values can be easily designed in the manufacturing process. The barrier properties can be manipulated by increasing crystallinity degree of the polymeric matrix or its modification with fillers, as well as incorporating PLLA/CD-lim films into multi-layer materials. The deteriorated optical properties may be leveled off by optimization of film thickness. Hence, there are many ways to balance undesired changes in compositions' appearance upon filler addition.

More importantly, composite formulations have superior barrier properties against UV light, which is crucial in designing food packaging films. The incorporation of CD-Lim also results in enhanced antibacterial properties. CD-Lim is an excellent antimicrobial agent against gram-positive bacteria and fungi. This outcome is critical as *Listeria monocytogenes*, the bacteria against which D-limonene is most effective, is hazardous and may cause a severe epidemiological problem.

The results presented in this manuscript prove that films made of PLLA and D-limonene encapsulated within β-cyclodextrin are able to extend shelf life and product quality and to prevent bacteria and fungi growth. The enhanced UV stability and antimicrobial properties balance slightly worsened performance properties proving that PLLA/CD-Lim composites are promising as active nourishment packages' materials. Most importantly, all used materials are biobased and biodegradable, therefore it is expected that the packaging can be easily disposed of after usage.

Acknowledgements

The authors wish to express their gratitude to Corbion for providing PLLA for the study. The research was supported by the National Science Centre in Poland under the Preludium project [UMO-2016/23/N/ST8/03799] and Poznan University of Technology [0613/SBAD/4710].

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

References

1. Wen, P.; Zhu, D.-H.; Feng, K.; Liu, F.-J.; Lou, W.-Y.; Li, N.; Zong, M.-H.; Wu, H. *Food Chem.* **2016**, *196*, 996.
2. Auras, R.; Harte, B.; Selke, S. *Macromol. Biosci.* **2004**, *4*, 835.
3. Ligaj, M.; Tichoniuk, M.; Cierpiszewski, R.; Foltynowicz, Z. *Coatings* **2020**, *10*, 156.
4. Tawakkal, I. S. M. A.; Cran, M. J.; Miltz, J.; Bigger, S. W. *J. Food Sci.* **2014**, *79*, R1477.
5. Burt, S. *Int. J. Food Microbiol.* **2004**, *94*, 223.
6. Mousavi Khaneghah, A.; Hashemi, S. M. B.; Limbo, S. *Food Bioprod. Process.* **2018**, *111*, 1.
7. Tariq, S.; Wani, S.; Rasool, W.; Shafi, K.; Bhat, M. A.; Prabhakar, A.; Shalla, A. H.; Rather, M. A. *Microb. Pathog.* **2019**, *134*, 103580.
8. Huang, T.; Qian, Y.; Wei, J.; Zhou, C. *Polymers (Basel).* **2019**, *11*, 560.
9. Falleh, H.; Ben Jemaa, M.; Saada, M.; Ksouri, R. *Food Chem.* **2020**, *330*, 127268.
10. Karami, Z.; Rezaeian, I.; Zahedi, P.; Abdollahi, M. *J. Appl. Polym. Sci.* **2013**, *129*, 756.
11. I, E. H.; Toska, V.; Baldisserotto, A.; Goci, E.; Vertuani, S. **2014**, 2.
12. Zhang, Z.; Vriesekoop, F.; Yuan, Q.; Liang, H. *Food Chem.* **2014**, *150*, 307.
13. Aggarwal, K. K.; Khanuja, S. P. S.; Ahmad, A.; Santha Kumar, T. R.; Gupta, V. K.; Kumar, S. *Flavour Fragr. J.* **2002**, *17*, 59.
14. Soković, M.; Glamočlija, J.; Marin, P. D.; Brkić, D.; Griensven, L. J. L. D. van *Molecules* **2010**, *15*, 7532.
15. Sun, J. **2007**, *12*, 259.
16. Chikhoune, A.; Hazzit, M.; Kerbouche, L.; Baaliouamer, A.; Aissat, K. *J. Essent. Oil Res.* **2013**, *25*, 300.
17. Settanni, L.; Palazzolo, E.; Guarrasi, V.; Aleo, A.; Mammina, C.; Moschetti, G.; Germanà, M. A. *Food Control* **2012**, *26*, 326.
18. Arrieta, M. P.; López, J.; Ferrándiz, S.; Peltzer, M. A. *Polym. Test.* **2013**, *32*, 760.
19. Arrieta, M. P.; López, J.; Hernández, A.; Rayón, E. *Eur. Polym. J.* **2014**, *50*, 255.
20. Del Nobile, M. A.; Conte, A.; Buonocore, G. G.; Incoronato, A. L.; Massaro, A.;

Panza, O. *J. Food Eng.* **2009**, *93*, 1.

21. López-de-Dicastillo, C.; Gallur, M.; Catalá, R.; Gavara, R.; Hernandez-Muñoz, P. *J. Memb. Sci.* **2010**, *353*, 184.
22. Mallardo, S.; De Vito, V.; Malinconico, M.; Volpe, M. G.; Santagata, G.; Di Lorenzo, M. L. *Eur. Polym. J.* **2016**, *79*, 82.
23. Dobrzyńska-Mizera, M.; Knitter, M.; Mallardo, S.; Del Barone, M. C.; Santagata, G.; Di Lorenzo, M. L. *Materials (Basel).* **2021**, *14*, 2569.
24. ISO 62 **2008**.
25. 12572, E. I. **2016**, 36.
26. PN-84/C-89100 **1984**.
27. Czarnecka-Komorowska, D.; Mencel, K. *Przem. Chem.* **2014**, *93*, 2.
28. ASTM E313 **2020**.
29. ISO 527-1 **2019**.
30. Supthanyakul, R.; Kaabbuathong, N.; Chirachanchai, S. *Polym. Degrad. Stab.* **2017**, *142*, 160.
31. Horváth, Z.; Gyarmati, B.; Menyhárd, A.; Doshev, P.; Gahleitner, M.; Varga, J.; Pukánszky, B. *RSC Adv.* **2014**, *4*, 19737.
32. Dobrzyńska-Mizera, M.; Dutkiewicz, M.; Sterzyński, T.; Di Lorenzo, M. L. *Eur. Polym. J.* **2016**, *85*, 62.
33. Petchwattana, N.; Naknaen, P.; Sanetuntikul, J.; Narupai, B. *Plast. Rubber Compos.* **2018**, *47*, 147.
34. Bonilla, J.; Atarés, L.; Vargas, M.; Chiralt, A. *Food Hydrocoll.* **2012**, *26*, 9.
35. Cele, H. M.; Ojijo, V.; Chen, H.; Kumar, S.; Land, K.; Joubert, T.; de Villiers, M. F. R.; Ray, S. S. *Polym. Test.* **2014**, *36*, 24.
36. Lizundia, E.; Vilas, J. L.; Sangroniz, A.; Etxeberria, A. *Eur. Polym. J.* **2017**, *91*, 10.
37. Astray, G.; Mejuto, J. C.; Morales, J.; Rial-Otero, R.; Simal-Gándara, J. *Food Res. Int.* **2010**, *43*, 1212.
38. Liu, B.; Zhu, X.; Zeng, J.; Zhao, J. *Food Res. Int.* **2013**, *54*, 691.
39. Ashrafi, A.; Jokar, M.; Mohammadi Nafchi, A. *Int. J. Biol. Macromol.* **2018**, *108*, 444.
40. Cocca, M.; Di Lorenzo, M. L.; Malinconico, M.; Frezza, V. *Eur. Polym. J.* **2011**, *47*, 1073.
41. Rhim, J.-W.; Park, H.-M.; Ha, C.-S. *Prog. Polym. Sci.* **2013**, *38*, 1629.
42. Brandelero, R. P. H.; Grossmann, M. V.; Yamashita, F. *Polímeros* **2013**, 0.
43. Bertuzzi, M. A.; Vidaurre, E. F. C.; Armada, M.; Gottifredi, J. C. **2007**, *80*, 972.

44. Maestrello, C.; Tonon, L.; Madrona, G.; Scapim, M. **2017**, *57*, 1393.
45. Siracusa, V. *Int. J. Polym. Sci.* **2012**, *2012*.
46. Karkhanis, S. S.; Stark, N. M.; Sabo, R. C.; Matuana, L. M. *Compos. Part A Appl. Sci. Manuf.* **2018**, *114*, 204.
47. Bastarrachea, L.; Dhawan, S.; Sablani, S. S. *Food Eng. Rev.* **2011**, *3*, 79.
48. Wu, F.; Misra, M.; Mohanty, A. K. *Prog. Polym. Sci.* **2021**, *117*, 101395.
49. Trifol, J.; Plackett, D.; Szabo, P.; Daugaard, A. E.; Giacinti Baschetti, M. Effect of Crystallinity on Water Vapor Sorption, Diffusion, and Permeation of PLA-Based Nanocomposites. *ACS Omega* **2020**, *5*, 15362–15369.
50. Yew, G. H.; Mohd Yusof, A. M.; Mohd Ishak, Z. A.; Ishiaku, U. S. *Polym. Degrad. Stab.* **2005**, *90*, 488.
51. Bula, K.; Klapiszewski, Ł.; Jesionowski, T. *Polym. Test.* **2019**, *77*, 105911.
52. Joo, M.; Auras, R.; Almenar, E. *Carbohydr. Polym.* **2011**, *86*, 1022.
53. Matle, I.; Mbatha, K. R.; Madoroba, E. *Onderstepoort J. Vet. Res.* **2020**, *87*.
54. Shakeri, F.; Shakeri, S.; Hojjatoleslami, M. *J. Food Sci.* **2014**, *79*, N697.
55. Kong, M.; Chen, X. G.; Liu, C. S.; Liu, C. G.; Meng, X. H.; Yu, L. J. *Colloids Surfaces B Biointerfaces* **2008**, *65*, 197.
56. Zhao, Y.; Chen, M.; Zhao, Z.; Yu, S. *Food Chem.* **2015**, *185*, 112.
57. Han, Y.; Sun, Z.; Chen, W. *Molecules* **2019**, *25*, 33.
58. Limm, W.; Hentges, S.; Begley, T.H. *Food Addit Contam.* **2006**, *23*, 738.
59. Fortunati, E.; Luzi, F.; Puglia, D.; Dominici; F.; Santulli, C.; Kenny, J. M.; Torre, L. *Eur. Polym. J.* **2014**, *56*, 77.
60. Scarfato, P.; Di Maio, L.; Incarnato, L. *J. Appl. Polym. Sci.* **2015**, *132*, 42597.