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Application of shellac for the development of probiotic formulations

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ABSTRACT

In this study, we have improved the enteric properties of shellac and developed probiotic formulations comprising this natural polymer. The effects of plasticizers such as glycerol and glyceryl triacetate, as well as water-soluble polymers such as sodium alginate, hydroxypropyl methylcellulose and polyvinylpyrrolidone on thermodynamic characteristics and coating properties of shellac were evaluated. The data indicate that glycerol showed the best plasticization effect. Hydroxypropyl methylcellulose and polyvinylpyrrolidone had superior miscibility with shellac compared to sodium alginate. Then, three fluid-bed dried bacterial species i.e., *Enterococcus faecium*, *Bifidobacterium bifidum* and *Lactobacillus reuteri*, were coated with formulations comprising different concentrations of shellac and additives. Coatings with shellac containing 5% glycerol or 5% sodium alginate or up to 20% [w/w] polyvinylpyrrolidone protected the microorganisms against acidic pH and provided the best release profile in simulated intestinal fluid. Moreover, these formulations maintained promising cell survival rates after four months of storage at 5 °C. *E. faecium* and *B. bifidum* showed more resistance to manufacturing process than *L. reuteri*.

1. Introduction

Probiotic products represent a significant growth area in the nutritional supplement industry, which is due to the increasing evidence of the health benefits associated with their application. However, the development of probiotic formulations is still a challenge because of the limited number of excipients approved for use in nutritional supplements, and insufficient cell stability during the manufacturing process and storage (Chan & Zhang, 2005; Laicher, Lorck, Grunenberg, Klemm, & Stanislaus, 1993; Lorck, Grunenberg, Jünger, & Laicher, 1997; Sultana et al., 2000). Searching for suitable materials of natural origin is therefore essential for increasing the number of approved additives, providing advanced end-product properties. Shellac is the purified product of the natural polymer Lac, the resinous secretion of the insect *Kerria lacca* (*Coccoidea*), which is a parasite found on several species of trees in Asian countries such as India, Thailand and China. The physico-chemical properties of shellac are variable depending on the strain of insect, host trees and refining methods (Buch, Penning, Wächtersbach, Maskos, & Langguth, 2009). Because of its natural origin, shellac is an acceptable coating material for food supplement products. In general, shellac possesses good resistance to gastric fluid, suggesting its use for enteric coating purposes. However, the low solubility of shellac in the intestinal fluid, especially in the case of enteric coating of hydrophobic substances limits its use as an enteric coating polymer (Limmatvapirat et al., 2004; Limmatvapirat, Limmatvapirat, Puttipipatkhachorn, Nuntanid, & Luangtana-anan, 2007; McGuire & Hagenmaier, 1996; Pearnchob & Bodmeier, 2003; Pearnchob, Dashevsky, & Bodmeier, 2004; Pearnchob, Dashevsky, Siepmann, & Bodmeier, 2003; Qussi & Suess, 2005).

This study aimed to improve the enteric coating properties of shellac in order to develop enteric coating formulations for probiotic microorganisms, including Bifidobacteria, Lactobacilli and Enterococci. Sodium alginate, hydroxypropyl methylcellulose (HPMC) and polyvinylpyrrolidone (PVP) were chosen as additional water-soluble polymers, and glycerol and glyceryl triacetate (GTA) as plasticizers. These additives were selected from the list of approved food additives published by the European Commission (EC) and the US Food and Drug Administration (FDA). The thermodynamic properties of shellac, and the effect of the mentioned polymers and plasticizers on these properties were evaluated. Furthermore, fluid-bed dried *Enterococcus faecium* M74, *Bifidobacterium bifidum* 12 and *Lactobacillus reuteri* ATCC 55730 were coated with the most suitable formulations comprising shellac and different additives. Given the sensitivity of probiotic microorganisms to the acidic pH of gastric juice and the necessity to release sufficient colony forming units (>6 log units/g or ml of product) in the intestine to achieve the probiotic effect (Lourens-Hattingh & Viljoen,

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2001), we have studied the enteric coating properties of formulations. In addition, the stability of microorganisms was investigated both after processing and after 4 months of storage at 5 °C.

2. Materials and methods

2.1. Materials

E. faecium M74, *B. bifidum* 12 and *L. reuteri* ATCC 55730 were provided by Medipharm (Sweden), Chr. Hansen A/S (Denmark), and BioGaia Biologics AB (Sweden), respectively. MRS broth and agar, kanamycin esculin azide agar and skim milk were provided by Merck, Austria. Reinforced clostridial broth and agar were purchased by Oxoid, Germany. HPMC (Pharmacoat®) and the aqueous ammonium salt of shellac (Marcoat 125®) were provided by Syntapharm, Germany. Marcoat 125® is prepared from the *Bysakhi* strain of shellac producing insect, which is cultivated in India. This shellac is produced by the solvent extraction method and is commercially available. Sodium alginate was provided by FMC, USA and polyvinylpyrrolidone (PVP, Kollidon 25®) by BASF, Austria. Microcrystalline cellulose (MCC) pellets were kindly donated by Pharmatrans Sanaq, Switzerland. Other chemicals were provided by Sigma–Aldrich, Austria.

2.2. Methods

2.2.1. Investigation of the coating properties of shellac

2.2.1.1. Swelling rate and weight loss of shellac films. Films were prepared from shellac or mixtures of shellac with water-soluble polymers in the ratios of 90 + 10, 80 + 20 and 70 + 30% [w/w] or mixtures of shellac with plasticizers in the ratios of 95 + 5, 90 + 10, 80 + 20 and 70 + 30% [w/w]. The mixtures were cast on aluminium frames and dried in an oven at 45 °C for 24 h. The resulting films had a thickness of 100–300 μm and a diameter of 15–17 mm. The miscibility of additives with shellac was evaluated based on the optical appearance of films. The swelling rate and weight loss of films with clear and homogeneous appearance were investigated after exposure to 50 ml buffer solutions with the pH of 1.2 for 2 h, or pH 6.8 and 7.5 for 3 h, in a horizontal shaker (Inova4000, Brunswick Sciences, USA) at 37 ± 0.5 °C and 80 rpm.

Swelling rates were calculated as follows:

$$\%\ \text{swelling rate} = [(We - Wo)/Wo] \times 100$$

We is the highest weight of the swollen film (g) during the exposure period of time, *Wo* is the original weight of the dry film (g).

For the calculation of weight loss the films were exposed to pH 1.2 for 2 h or pH 6.8 and 7.5 for 3 h. Afterwards, they were dried at 45 ± 2 °C until a constant weight was obtained. The % weight loss was calculated as follows:

$$\%\ \text{weight loss} = [(Wo - Wd)/Wo] \times 100$$

Wo is the original weight of the dry film (g), *Wd* is the weight of the film (g) after removal from the medium and drying at 45 °C.

2.2.1.2. Thermal analysis of shellac films. Melting temperature (T_m) and enthalpy change (ΔH) of films consisting of shellac or different ratios of shellac and plasticizer were determined using differential scanning calorimetry (DSC 7, PerkinElmer, USA) (Table 1). Approximately 10 mg of each dried sample was sealed in aluminium pans and investigated under a N_2 atmosphere ($n = 3$). The samples were heated from 20 to 200 °C at a rate of 10 °C/min.

Table 1
Thermodynamic properties of shellac films containing different levels of additives.

Additive (w/w, based on the mass of shellac)	T_m (±sd) [°C]	ΔH (±sd) [J/g]
None	73.87 ± 1.58	16.92 ± 0.51
Glycerol 5%	59.33 ± 0.20	11.06 ± 0.30
Glycerol 10%	57.20 ± 0.22	10.45 ± 0.35
Glycerol 20%	56.03 ± 0.52	7.65 ± 0.22
Glycerol 30%	49.36 ± 1.35	3.47 ± 0.10
Glyceryltriacetate 5%	62.10 ± 0.10	13.77 ± 0.20
Glyceroltriacetate 10%	54.80 ± 0.32	13.10 ± 0.22
Glyceroltriacetate 20%	55.66 ± 0.57	11.18 ± 0.66
Glyceroltriacetate 30%	60.50 ± 0.10	13.40 ± 0.15

2.2.2. Development of enteric coating formulations

The development of enteric coating formulations was carried out in two steps.

The first step consisted of the drying of bacteria and the second, of the enteric coating of dried microorganisms with different formulations containing shellac. The drying and coating processes were performed in a laboratory fluid bed apparatus (GPCG 1.1, Glatt, Germany) with a bottom spray (Wurster) design and a modified outlet filter system (Innojet, Germany). The parameters for drying as well as for coating processes were adjusted as follows:

$$\text{Product bed temperature} = 37 \pm 2\ ^\circ\text{C}$$

$$\text{Spraying rate} = 7 \pm 2\ \text{g/min}$$

The atomizing air pressures of 1.5 and 1.7 bar were used for drying and coating processes, respectively.

2.2.2.1. Drying of probiotic microorganisms. The microorganisms were cultured in their appropriate media and harvested at the beginning of the stationary phase. *E. faecium* M74 and *L. reuteri* ATCC 55730 were cultured in MRS broth. Reinforced clostridial broth was used for the culturing of *B. bifidum* 12. Based on the results of preliminary studies, concentrations of 50% sucrose and 50% skim milk according to the wet mass of cells were added to the harvested bacteria as protectants. Afterwards, the mixture of bacteria and protectant was suspended in distilled water. The drying process was undertaken in the fluid bed apparatus. The MCC pellets were used as carrier. Batches of 500 g MCC pellets were layered with the suspensions of either *E. faecium* M74 or *L. reuteri* ATCC 55730 or *B. bifidum* 12. Thereafter, the layered pellets were used for the enteric coating.

2.2.2.2. Enteric coating of layered pellets with probiotic microorganisms 2.2.2.2.1. Investigation of the ability of coatings to protect the probiotic microorganisms against the simulated gastric fluid (SGF). Listed in Table 2 are the concentrations of shellac and of the mentioned additives which were miscible, resulted in clear films and were chosen for coating the microorganisms. *E. faecium* M74 was chosen as model strain, because of its robustness. The ability of the formulations listed in Table 2 and the required coating level for the protection of this strain against the low pH of gastric juice was firstly investigated. The pellets layered with *E. faecium* M74 were coated to achieve coating levels of 3, 4.5 or 6 mg/cm^2. Afterwards, the viability of free and coated microorganisms in simulated gastric fluid (SGF, HCl 0.1 N, pH 1.2) was examined. The tests were performed according to USP XXXI, using the paddle method (Pharma Test, Germany) at 100 rpm, 37 °C with the modified amount of 200 ml of SGF. Coated or free pellets (1 ± 0.05 g) layered with *E. faecium* M74 were exposed to SGF for 2 h ($n = 3$). Afterwards, 0.1 ml of the SGF was spread onto Kanamycin esculin azide agar plates ($n = 3$). Additionally, the pellets were removed from SGF and washed with phosphate buffer pH 6.8, in order to neutralize the SGF. The coatings were crushed with a glass bar

Table 2
Survival of *E. faecium* M74, coated with different formulations containing shellac, after 2 h exposure to simulated gastric fluid (SGF).

Trials	Enteric coatings containing shellac	CFU of *E. faecium* M74/g of pellets before exposure to SGF (±sd)	CFU of *E. faecium* M74/g of pellets after exposure to SGF (±sd)	Fold change in CFU of *E. faecium* M74/g of pellets (after exposure to SGF/before exposure to SGF)
1	Free *E. faecium* M74	1.35E+07 (±22%)	–*	0
2	100% [w/w] shellac (coating level: 6 mg/cm^2)	2.77E+07 (±13.5%)	2.37E+07 (±15.1%)	0.85
3	100% [w/w] shellac (coating level: 4.5 mg/cm^2)	2.15E+07 (±15.7%)	2.42E+07 (±11.2%)	1.12
4	100% [w/w] shellac (coating level: 3 mg/cm^2)	2.86E+07 (±16.2%)	3.10E+07 (±8.54%)	1.08
5	95% + 5% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	5.87E+08 (±8%)	5.80E+08 (±43.7%)	0.99
6	90% + 10% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	2.48E+07 (±15.4%)	1.70E+07 (±10.6%)	0.68
7	80% + 20% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	1.30E+07 (±19%)	1.20E+07 (±8%)	0.92
8	95% + 5% [w/w] shellac + GTA (coating level: 3 mg/cm^2)	6.55E+07 (±28.4%)	8.30E+06* (±24%)	0.13
9	90% + 10% [w/w] shellac + GTA (coating level: 3 mg/cm^2)	1.40E+09 (±20%)	7.90E+08* (±7.57%)	0.6
10	90% + 10% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	4.56E+07 (±31.7%)	5.88E+07 (±24%)	1.29
11	80% + 20% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	6.50E+07 (±28.9%)	2.07E+07* (±38.6%)	0.32
12	90% + 10% [w/w] shellac + HPMC (coating level: 3 mg/cm^2)	2.33E+07 (±24.7%)	3.23E+07 (±30.23%)	1.38
13	80% + 20% [w/w] shellac + HPMC (coating level: 3 mg/cm^2)	2.17E+07 (± 35.3%)	1.15E+04* (±45.5%)	0.00005
14	95% + 5% [w/w] shellac + sodium alginate (coating level: 3 mg/cm^2)	2.06E+07 (± 19.1)	7.30E+06* (±40%)	0.35
15	95% + 5% [w/w] shellac + sodium alginate (coating level: 4.5 mg/cm^2)	6.90E+07 (±21%)	1.67E+07* (±16%)	0.24
16	90% + 10% [w/w] shellac + sodium alginate (coating level: 4.5 mg/cm^2)	4.00E+07 (±32%)	–*	0

* p-value < 0.05 (significance of change in CFU/g of pellets after 2 h exposure to pH 1.2).

and the viability of cells in the films was determined by spreading onto agar plates (n = 3). The viability of cells was expressed as colony forming units per gram of pellets (CFU/g).

2.2.2.3. Release of bacteria in simulated intestinal fluid (SIF). The coatings which protected the *E. faecium* M74 against SGF are listed in Table 3. The release of microorganisms from these coatings into SIF was investigated performing dissolution tests according to USP XXXI with modified amounts of medium. This means using 200 ml SGF and SIF, respectively (n = 3).

First, 1 ± 0.05 g of coated pellets were exposed to SGF for 2 h and subsequently to SIF with the pH of 6.8 or 7.5, for 3 h. The pH values of 6.8 and 7.5 were chosen to represent the milieu of the entire small intestine. Phosphate buffer was used as SIF according to USP XXXI. During the exposure of pellets to SIF, 0.1 ml of the SIF was removed at 60 min intervals and spread onto Kanamycin esculin azide agar plates (n = 3). After 3 h the pellets were removed, washed with phosphate buffer and crushed with a glass bar and the viability of cells in the films was determined by spreading onto agar plates (n = 3).

The formulations which protected the *E. faecium* M74 against SGF and served for appropriate release of microorganisms in SIF were chosen for the coating of *L. reuteri* ATCC 55730 and *B. bifidum* 12. The same procedure as described for *E. faecium* M74 was used, with the exception of utilizing MRS agar and reinforced clostridial agar for spreading of *L. reuteri* ATCC 55730 and *B. bifidum* 12, respectively, under anaerobic conditions.

2.2.3. Stability of the encapsulated cells

After each coating process, samples with optimal enteric properties were sealed in two layer aluminium bags and stored for 4 months at 5 °C. The viability of cells was assessed by crushing the coatings with a glass bar and spreading onto appropriate agar plates.

2.2.4. Data analysis

Data were analyzed using the statistical analysis package of Microsoft Excel 2003. Student's t-tests were performed and p-values < 0.05 were considered as significant.

Table 3
Release of *E. faecium* M74, coated with different formulations containing shellac, in simulated intestinal fluid (SIF).

Trials	Enteric coatings containing shellac	CFU of *E. faecium* M74/g of pellets (±sd)	CFU of *E. faecium* M74/g of pellets released in SIF (±sd)
1	100% [w/w] shellac (coating level: 3 mg/cm^2)	2.86E+07 (±16.2%)	5.19E+02 (±60%)
2	95% + 5% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	5.87E+08 (±8%)	2.13E+07 (±25%)
3	90% + 10% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	2.48E+07 (±15.4%)	1.30E+03 (±40%)
4	80% + 20% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	1.30E+07 (±19%)	2.80E+03 (±35%)
5	95% + 5% [w/w] shellac + GTA (coating level: 3 mg/cm^2)	6.55E+07 (±28.4%)	5.00E+05 (±45%)
6	90% + 10% [w/w] shellac + GTA (coating level: 3 mg/cm^2)	1.40E+09 (±20%)	2.96E+02 (±60%)
7	90% + 10% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	4.56E+07 (±31.7%)	2.60E+06 (±30%)
8	80% + 20% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	6.50E+07 (±28.9%)	2.53E+06 (±28%)
9	90% + 10% [w/w] shellac + HPMC (coating level: 3 mg/cm^2)	2.33E+07 (±24.7%)	2.50E+05 (±28%)
10	80% + 20% [w/w] shellac + HPMC (coating level: 3 mg/cm^2)	2.17E+07 (±35.3%)	2.50E+02 (±35%)
11	95% + 5% [w/w] shellac + sodium alginate (coating level: 3 mg/cm^2)	2.06E+07 (±19.1%)	3.50E+06 (±38%)

The efficacy of the above-described formulations for the enteric encapsulation of lyophilised or spray dried probiotic microorganisms is a further important aspect, which is in progress.

3. Results

3.1. Investigation of the coating properties of shellac

3.1.1. Weight loss and swelling rate of films

The miscibility of water-soluble polymers and plasticizers with shellac depended on the chemical structure and concentration of these additives. Films prepared of up to 20% [w/w] HPMC or PVP and 80% [w/w] shellac were clear. Sodium alginate was poorly miscible with shellac, as mixtures containing ⩾10% [w/w] sodium alginate tended to separate in two phases and resulted in turbid films. Glycerol was very well miscible with shellac. Increasing the concentration of glycerol to 30% [w/w] resulted in clear films with improved elasticity. In contrast, the addition of more than 10% [w/w] GTA to shellac resulted in turbid films.

Figs. 1–4 demonstrate the weight loss and swelling rate of films consisting of mixtures of shellac and polymer or shellac and plasticizer after 2 h exposure to pH 1.2 (Panel a) or 3 h exposure to pH 6.8 and 7.5 (Panels b and c, respectively). The weight loss of films represents their solubility in different media. Investigation of the solubility of films consisting of 100% [w/w] shellac showed the insolubility of these films at acidic pH and their low solubility of 8.87 ± 1.1% [w/w] at pH 6.8 (Fig. 1). Investigation of the swelling rate of films in different media (Figs. 3 and 4) confirmed the low swelling rate of 100% [w/w] shellac films at pH 1.2 (8.6 ± 2.19% [w/w]). In contrast, the swelling rate of these films was more than 150% [w/w] after exposure to pH 6.8 and almost 400% (w/w) after exposure to pH 7.5 (Fig. 3b and c). The insolubility of shellac and its low swelling rate in acidic pH as well as its high capacity of fluid uptake at pH 6.8 and 7.5, make this polymer suitable for use as enteric coating polymer. However, its low solubility in intestinal juice is a problem for enteric release of hydrophobic active ingredients. In order to investigate the effect of additives on the solubility and swelling rate of shellac films at pH 1.2 as well as at pH 6.8 and 7.5, films were prepared containing shellac and above-described water-soluble polymers and plasticizers. Concentrations, which resulted in a clear film, were chosen. This means 10% [w/w] sodium alginate, 10% and 20% [w/w] HPMC or PVP and 5%, 10% and 20% [w/w] glycerol or GTA, based on the mass of shellac.

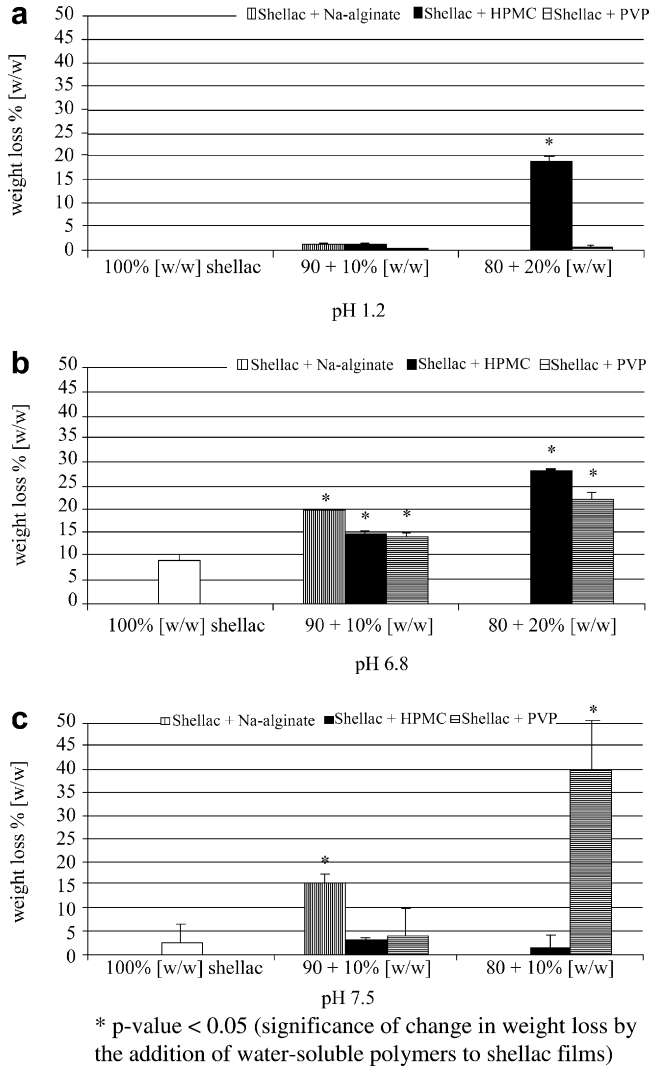

Fig. 1. Weight loss of films consisting of different levels of shellac and water-soluble polymers after: (a) 2 h exposure to pH 1.2, (b) 3 h exposure to pH 6.8 and (c) 3 h exposure to pH 7.5.

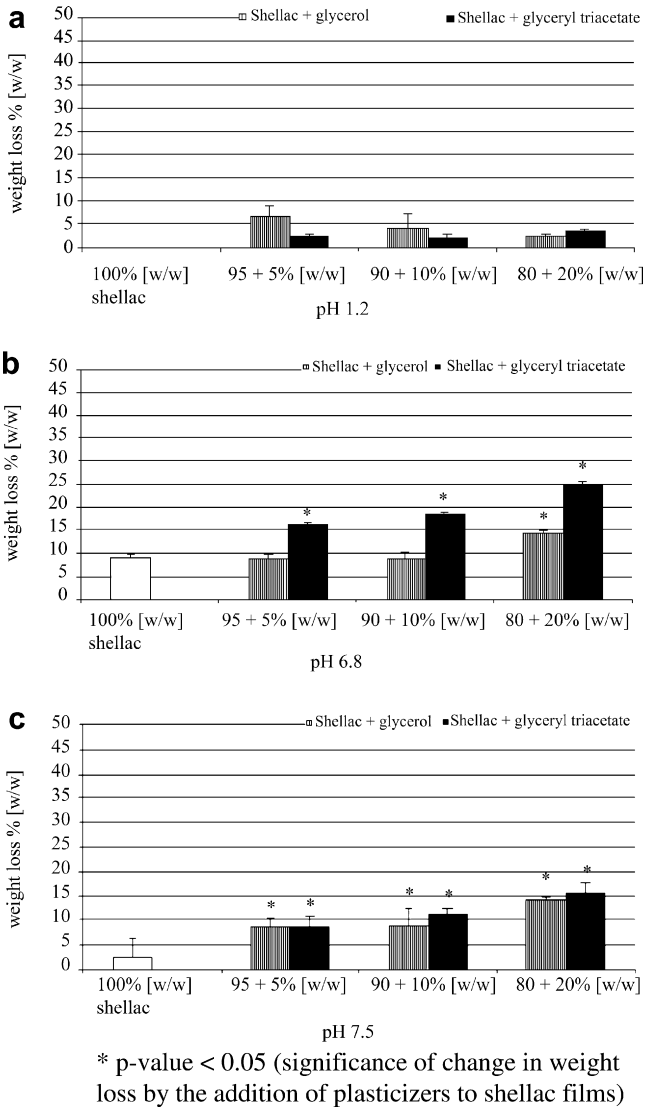

Fig. 2. Weight loss of films consisting of different levels of shellac and plasticizer after: (a) 2 h exposure to pH 1.2, (b) 3 h exposure to pH 6.8 and (c) 3 h exposure to pH 7.5.

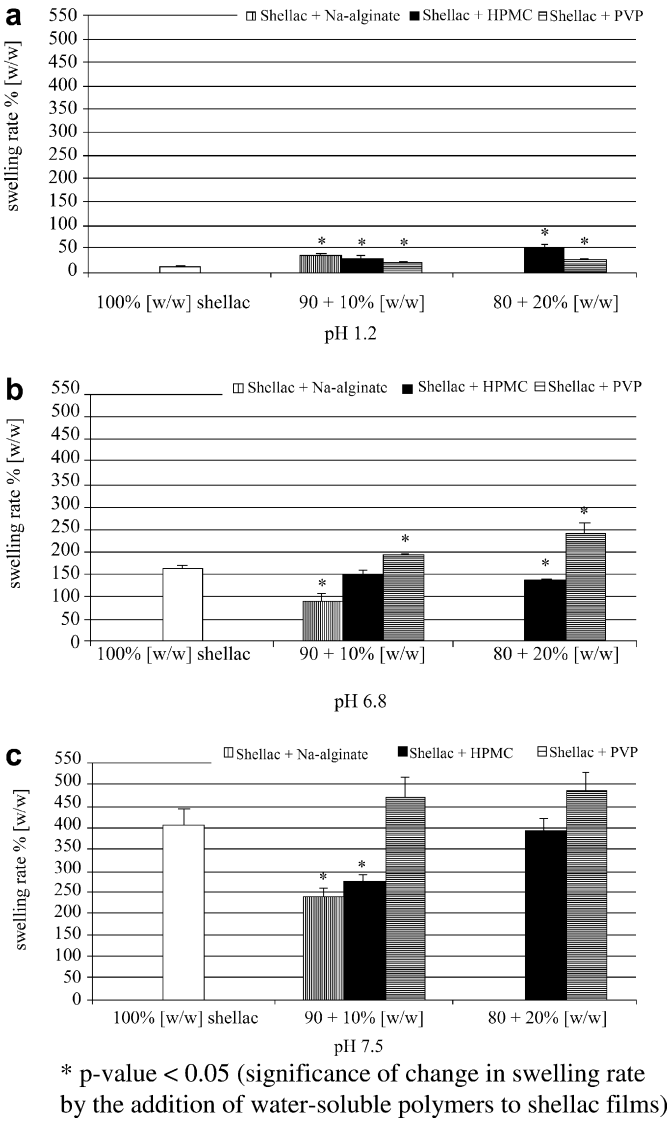

Fig. 3. Swelling rate of films consisting of different levels of shellac and water-soluble polymers during: (a) 2 h exposure to pH 1.2, (b) 3 h exposure to pH 6.8 and (c) 3 h exposure to pH 7.5.

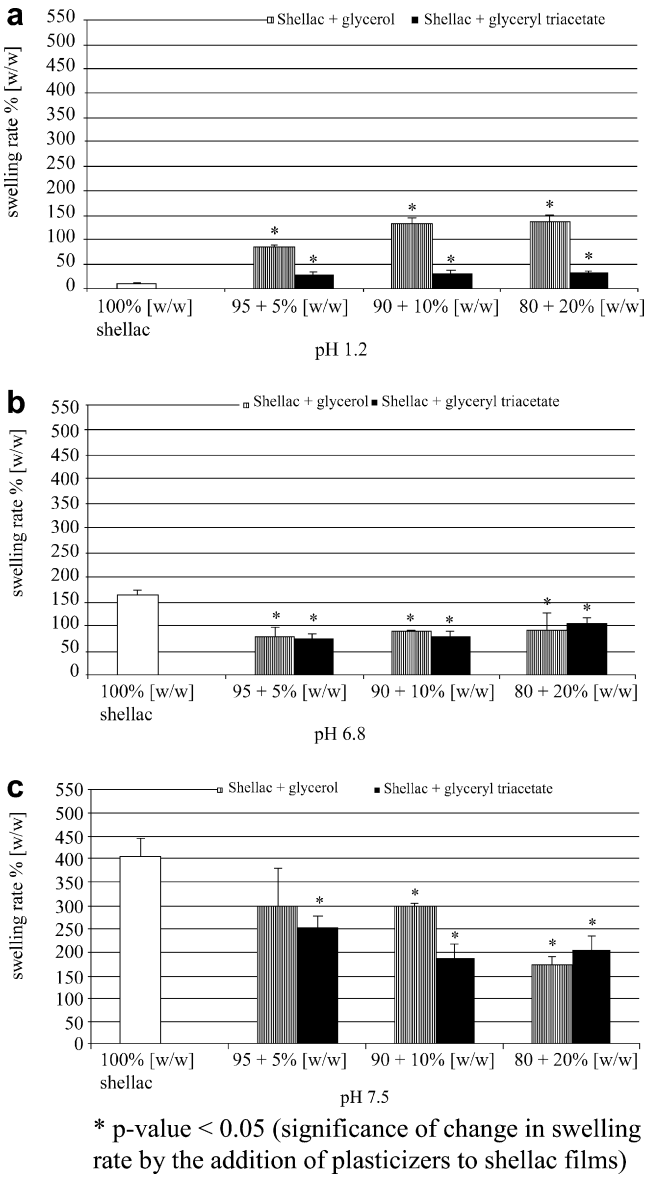

Fig. 4. Swelling rate of films consisting of different levels of shellac and plasticizer during: (a) 2 h exposure to pH 1.2, (b) 3 h exposure to pH 6.8 and (c) 3 h exposure to pH 7.5.

The addition of water-soluble polymers to shellac had diverse effects on the solubility and swelling rate of films. Generally, addition of 10% [w/w] water-soluble polymer to shellac had a negligible effect on the solubility of films at pH 1.2, but resulted in improved solubility at pH 6.8. Addition of 10% [w/w] sodium alginate particularly, affected the solubility of films in both pH 6.8 and 7.5. As can be seen from Fig. 1b and c, the solubility of films containing 10% [w/w] sodium alginate were 20% and 15% [w/w] at pH 6.8 and 7.5, respectively, and these were higher than the solubility of films containing 10% [w/w] HPMC or PVP at pH 6.8 and 7.5. Increasing the concentration of HPMC to 20% [w/w] in films resulted in increased solubility rates at both pH 1.2 and 6.8, whereas the solubility of films at pH 7.5 was not affected (Fig. 1). The solubility of these films was almost 19% [w/w] after 2 h exposure to pH 1.2. Consequently, as will be presented in the next section, the formulations containing 20% [w/w] HPMC could not protect the probiotic microorganisms against the SGF. In contrast, the addition of 20% [w/w] PVP to shellac resulted in barely increased solubility of films at pH 1.2. However, the solubility of these films considerably increased in both pH 6.8 (22.04 ± 1.5% [w/w]) and pH 7.5 (39.43 ± 11.5% [w/w]). Due to the very low solubility of films containing sodium alginate or PVP at pH 1.2 and their improved solubility at pH 6.8 or 7.5, their suitability as enteric coating formulations could be predicted.

Regarding the swelling rate, the addition of water-soluble polymers to shellac resulted in the general increasing of the swelling rate of films at pH 1.2. The swelling rate of films consisting of 100% [w/w] shellac in acidic pH was 8.6 ± 2.19% [w/w]. Addition of 10% [w/w] sodium alginate, HPMC or PVP resulted in the increased swelling rate of 32.67 ± 4.5%, 26.78 ± 4.4% or 17.31 ± 1.2% [w/w], respectively (Fig. 3a). However, at pH 6.8 a significant increase of the swelling rate could only be observed by the addition of PVP to shellac. Films consisting of 10% [w/w] PVP had a swelling rate of 193.62 ± 2.78% [w/w] and increasing the concentration of PVP to 20% [w/w] resulted in the increased swelling rate of 242.17 ± 20% [w/w] at pH 6.8 (Fig. 3b). The swelling rates of films containing 10% [w/w] sodium alginate at pH 6.8 and 7.5 were less than the swelling rate of films containing 100% [w/w] shellac. The addition of HPMC resulted in films with either equal or lesser

swelling rates than the swelling rate of 100% [w/w] shellac films. The reason might be the increased solubility of films containing sodium alginate or HPMC at pH 6.8 and 7.5.

Using 5% [w/w] glycerol as plasticizer resulted in the increased solubility of films at pH 1.2 and 7.5 (6.58 ± 2% and 8.55 ± 1.5% [w/w], respectively). Increasing the concentration of glycerol to 20% [w/w] had different effects on the solubility of films. As can be seen in Fig. 2, the solubility of these films decreased at pH 1.2 (2.2 ± 0.83% [w/w]), while there was a significant increase of solubility at pH 6.8 and 7.5 (14.26 ± 0.64% and 14.27 ± 0.4% [w/w], respectively), predicting their proper enteric coating behaviour. The addition of glycerol also had the most considerable effect on the swelling rate of films. Increasing the concentration of glycerol in films resulted in increased swelling rates at pH 1.2. In contrast, the swelling rate of films was decreased at pH 6.8 and 7.5 (Fig. 4). The addition of GTA to shellac had a negligible effect on the solubility and swelling rate of films at pH 1.2. However, compared to the 100% [w/w] shellac films, the films containing GTA had an improved solubility at pH 6.8 and 7.5, and their swelling rate was significantly decreased.

3.1.2. Thermal analysis of shellac films

The effect of different concentrations of the chosen two plasticizers, i.e. Glycerol and GTA, on the melting temperature (T_m) and enthalpy change (ΔH) of shellac films are listed in Table 1. The unplasticized shellac films possess a T_m of 73.87 ± 1.58 °C and a ΔH of 16.92 ± 0.51 J/g (Table 1). Generally, the addition of plasticizer reduced both T_m and ΔH of films to different extents. The plasticizing effect of glycerol could be observed at concentrations of 5% [w/w], as the T_m of films was reduced to 59.33 ± 0.20 °C. Using up to 30% glycerol based on the mass of shellac resulted in the reduction of T_m and ΔH to 49.36 ± 1.35 °C and 3.47 ± 0.10 J/g, respectively. In the case of GTA, films consisting of 5% GTA based on the mass of shellac showed a T_m of 62.1 ± 0.10 °C and ΔH of 13.77 ± 0.20 J/g. Increasing the concentration of GTA to 10% [w/w] resulted in the reduction of T_m to 54.80 ± 0.32 °C. However, concentrations above 10% [w/w] GTA had no further plasticizing effect on shellac films (Table 1). This was in agreement with the observation that amounts over 10% [w/w] were not miscible with shellac.

3.2. Enteric coating of layered pellets with probiotic bacteria

3.2.1. Investigation of the ability of coatings to protect E. faecium M74 against the simulated gastric fluid (SGF)

The ability of different coatings for protection of *E. faecium* M74 against the SGF is listed in Table 2. The ratio of CFU/g of pellets before exposure to SGF to the CFU/g of pellets after exposure has been presented as fold change in CFU. Preparations showing decreases of at least 1 log unit CFU after exposure to pH 1.2 (fold change $\leqslant 0.1$) were considered as not resistant to SGF.

The investigations showed that a coating level of 3 mg/cm^2 with 100% [w/w] shellac protected the *E. faecium* M74 against the SGF. As can be observed from Table 2, the fold change in CFU of *E. faecium* M74/g of pellets coated with a level of 3 mg/cm^2 shellac was 1.08. Keeping the coating level of 3 mg/cm^2 constant, the gastric fluid resistance of different formulations containing shellac and additives has been investigated.

In the case of mixtures of shellac and water-soluble polymers, formulations consisting of 10% [w/w] HPMC or PVP with 90% [w/w] shellac were resistant to SGF. There were no significant changes in CFU of microorganisms in the shell after 2 h exposure to pH 1.2. Using 20% [w/w] PVP, the coatings were still resistant to SGF, although the fold change in CFU (0.32) was less than the fold change when using 10% [w/w] PVP (1.29). In contrast, coatings containing 20% [w/w] HPMC could not protect the *E. faecium* M74 at pH 1.2 (Table 2). Using sodium alginate as additive, the preparations with a concentration of 5% [w/w] were resistant to SGF. Increasing the concentration of sodium alginate, the coatings were no more resistant, even not by increasing the coating level to 4.5 mg/cm^2 (Table 2).

Generally, coatings consisting of shellac and plasticizer were resistant to SGF. However, after 2 h exposure to SGF the values of fold changes in CFU of coatings containing glycerol were higher than coatings containing GTA, a reason for the superior protecting effect of films containing glycerol (Table 2).

Regarding the fold changes in CFU after 2 h exposure of different coatings to pH 1.2, it could be concluded that following coatings protect *E. faecium* M74 against SGF:

- 100% [w/w] shellac.
- 5% [w/w] glycerol, GTA or sodium alginate.
- 10% [w/w] glycerol, GTA, PVP or HPMC.
- 20% [w/w] glycerol or PVP.

3.2.2. Release of E. faecium M74 in simulated intestinal fluid (SIF)

The release properties of resistant formulations to SGF were separately investigated during 3 h exposure to pH 6.8 and 7.5. The released number of CFU/g of pellets was determined in 1 h intervals. In all formulations, the released number of CFU/g of pellets after 3 h exposure to pH 6.8 was the same as after 1 h exposure to pH 7.5. These data are presented in Table 3 in the right hand column as "CFU of *E. faecium* M74/g of pellets released in SIF".

Even though the films consisting of 100% [w/w] shellac were resistant to SGF (Table 2), it can be observed from Table 3 that there was no adequate release of microorganisms from these films after exposure to SIF (5.19E+02 CFU/g of pellets, Table 3).

Among the films consisting of shellac and water-soluble polymers with gastric fluid resistance properties, the films containing 5% [w/w] sodium alginate or 10% and 20% [w/w] PVP provided the best release rate of microorganisms in SIF. These results were in agreement with the improved solubility and swelling rate of these films at pH 6.8 and 7.5 (Table 3, Figs. 1 and 3).

Comparison of the release of CFU/g of pellets from coatings containing 5% [w/w] glycerol with coatings containing 5% [w/w] GTA showed the superior release properties of coatings containing glycerol. Interestingly, increasing the concentration of both plasticizers in the formulation resulted in a considerable reduction of the release of *E. faecium* from coatings (Table 3). This result was in agreement with the observation that increased amounts of plasticizer in the polymeric coating provide a greater degree of coalescence and consequently resulted in a reduced rate of drug release (Goodhart, Harris, Murthy, & Nesbitt, 1984; Hutchings & Sakr, 1994).

3.2.3. Enteric coating of L. reuteri ATCC 55730 and B. bifidum 12

According to the above results, we have selected the most suitable formulations comprising shellac and 5% [w/w] glycerol or Na-alginate or shellac and 10% [w/w] or 20% [w/w] PVP for the enteric coating of layered pellets with *L. reuteri* ATCC 55730 and *B. bifidum* 12. The formulation comprising 10% [w/w] HPMC was used as well. In the case of *L. reuteri* ATCC 55730, a coating level of 3 mg/cm^2 was applied for all formulations. Coatings comprising shellac and 5% [w/w] glycerol, shellac and 10% [w/w] HPMC or 10% [w/w] PVP could protect the microorganisms against the SGF and the release of the microorganisms in the SIF was adequate (Table 4).

In the case of *B. bifidum* 12, the application of 3 mg/cm^2 coating, regardless of the formulation's composition, could not protect the microorganisms against SGF. Therefore, a coating level of 4.5 mg/cm^2 was applied for all formulations, which provided enough resistance against pH 1.2. Furthermore, the release of colony forming units of *B. bifidum* 12 from these coatings in the SIF

Table 4
Enteric coating properties of *L. reuteri* ATCC 55730 and *B. bifidum* 12 coated with different formulations containing shellac.

Trials	Enteric coatings containing shellac	CFU of MOs[a]/g of pellets before exposure to SGF (±sd)	CFU of MOs[a]/g of pellets after exposure to SGF (±sd)	Fold change in CFU of MOs[a]/g of pellets (after exposure to SGF/ before exposure to SGF)	CFU of MOs[a]/g of pellets released in SIF (±sd)
L. reuteri ATCC 55730					
1	Free *L. reuteri* ATCC 55730	1.30E+07 (±10.2%)	–*	0	–
2	95% + 5% [w/w] shellac + glycerol (coating level: 3 mg/cm^2)	3.89E+08 (±2.98%)	1.61E+08* (±7.91%)	0.41	1.50E+06 (±35.5%)
3	90% + 10% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	9.80E+08 (±5.77%)	6.15E+08* (±10.35%)	0.63	5.00E+05 (±38.5%)
4	80% + 20% [w/w] shellac + PVP (coating level: 3 mg/cm^2)	4.70E+08 (±6.02%)	2.95E+07* (±2.40%)	0.06	1.80E+06 (±23.33%)
5	90% + 10% [w/w] shellac + HPMC (coating level: 3 mg/cm^2)	2.47E+08 (±8.59%)	1.20E+08* (±4.14%)	0.48	3.14E+06 (±27.0%)
6	95% + 5% [w/w] shellac + sodium alginate (coating level: 4.5 mg/cm^2)	9.10E+07	1.30E+05*	0.0014	–
B. bifidum 12					
1	Free *B. bifidum* B12	1.22E+07 (±12.2%)	–*	0	–
2	95% + 5% [w/w] shellac + glycerol (coating level: 4.5 mg/cm^2)	1.89E+08 (±7.17%)	1.14E+08* (±9.17%)	0.604	2.50E+06 (±38.04%)
3	90% + 10% [w/w] shellac + PVP (coating level: 4.5 mg/cm^2)	1.59E+08 (±3.93%)	5.02E+08* (±19.80%)	3.16	1.17E+06 (±27%)
4	80% + 20% [w/w] shellac + PVP (coating level: 4.5 mg/cm^2)	2.70E+08 (±10.47%)	6.90E+08 (±6.15%)	2.56	2.30E+07 (±35%)
5	90% + 10% [w/w] shellac + HPMC (coating level: 4.5 mg/cm^2)	6.97E+08 (±11.00%)	1.13E+07* (±6.70%)	1.63	5.30E+06 (±33%)
6	95% + 5% [w/w] shellac + sodium alginate (coating level: 4.5 mg/cm^2)	4.37E+08 (±10.45%)	1.68E+08* (±9.51%)	0.38	1.32E+07 (±33%)

* p-value < 0.05 (significance of change in CFU/g of pellets after 2 h exposure to pH 1.2).
[a] MOs = microorganisms.

Table 5
Viability of enteric coated microorganisms stored at 5 °C for 4 months.

Trials	Enteric coatings containing shellac	Fold change in CFU of MOs[a]/g of pellets (after 4 months 5 °C/after coating)		
		E. faecium M74 (coating level: 3 mg/cm^2)	*L. reuteri* ATCC 55730 (coating level: 3 mg/cm^2)	*B. bifidum* 12 (coating level: 4.5 mg/cm^2)
1	95% + 5% [w/w] shellac + glycerol	0.98	0.98	0.98
2	90% + 10% [w/w] shellac + PVP	0.72*	0.89	0.98
3	80% + 20% [w/w] shellac + PVP	0.62*	0.96	0.65
4	90% + 10% [w/w] shellac + HPMC	0.42*	0.83	0.53*
5	95% + 5% [w/w] shellac + sodium alginate	0.58*	–	0.34*

* p-value < 0.05 (significance of change in CFU/g of pellets after 4 months of storage at 5 °C).
[a] MOs = microorganisms.

was comparable with the results of the release of *E. faecium* M74 from the same coatings (Tables 3 and 4).

3.3. Stability of the encapsulated cells

The microorganisms coated with those formulations showing the best enteric properties were stored at 5 °C for 4 months, and cell viability was determined at the end of the storage period (Table 5). The ratio of CFU/g of pellets after 4 months storage at 5 °C to the CFU/g of pellets immediately after coating are presented as fold changes in CFU in Table 5. The formulations with a reduction of maximally 1 log unit CFU/g of pellets are considered as stable, which was consequently calculated as fold change equal or more than 0.1.

Generally, all formulations were stable during 4 months' storage at 5 °C, as the fold changes in CFU/g of pellets were overall more than 0.1.

4. Discussion

Due to the natural origin of shellac and its approved status as food additive by FDA as well as the good resistance of this polymer to gastric fluid, shellac is an attractive candidate for the use as an excipient for enteric coating of probiotics. The disadvantage of this polymer is its low solubility in intestinal fluid. The undertaken investigations in this study showed that the films consisting of 100% [w/w] shellac were rigid and possessed a T_m of 73.87 ± 1.58 °C and a ΔH of 16.92 ± 0.51 J/g (Table 1). Although these films were able to protect the microorganisms against the SGF, there was no adequate release of microorganisms in SIF. These results were in agreement with the results of investigation of solubility and swelling rate of 100% [w/w] shellac films (insolubility and the low swelling rate at pH 1.2 as well as the low solubility at pH 6.8, see Figs. 1 and 3), and also in agreement with the results of other investigators (Limmatvapirat et al., 2004, 2007; Pearnchob, Dashevsky, & Bodmeier, 2004).

In the present study, the thermodynamic as well as the enteric coating properties of shellac were improved by the addition of plasticizers and water-soluble polymers.

Respecting to the high release rate of microorganisms from coatings containing water-soluble polymers in SIF as well as the general improved solubility of films containing these polymers at pH 6.8, it could be expected that these polymers have a function as pore-formers in the films. The best release rate of *E. faecium*

M74 and *B. bifidum* 12 in SIF could be achieved by using coatings containing 5% [w/w] sodium alginate or 10% or 20% [w/w] PVP. These results were in agreement with the increased solubility of films containing sodium alginate and the increased swelling rate of films containing PVP at both pH 6.8 and 7.5. The high swelling rate of films containing PVP enabled the diffusion of microorganisms through the films.

Generally, the efficiency of a plasticizer is related to its chemical structure defining the interaction between its functional groups with those of the polymer and to the amount of plasticizer in the film (Gutierrez-Rocca & McGinity, 1994; Wu & McGinity, 1999). The plasticizer must be miscible with the polymer and must cohere by similar intermolecular forces. The specific interactions are hydrogen-bond formation and charge-transfer, which can alter the thermodynamic properties of a polymer significantly (Tarvainen, Sutinen, Somppi, Paronen, & Poso, 2001). The enteric coating properties of films containing glycerol were superior to films containing GTA. Moreover, glycerol had a better plasticizing effect on shellac films. Glycerol and GTA possess different types of functional groups for hydrogen bonding and approximate molecular weights of 92 and 218, respectively. Glycerol is a triol with small molecular volume. Its plasticizing effect on shellac can be explained by diffusion of glycerol molecules between the shellac chains and by the formation of hydrogen bonds. This interaction results in cross linking of shellac molecules and in diminished formation of crystallites (Lim & Wan, 1994), which can be reasons for the reduction of T_m of films containing shellac and glycerol. Moreover, increasing the concentration of glycerol in films resulted in the improvement of their elasticity, which also argues for the miscibility of glycerol with shellac.

GTA has a larger molecular volume than glycerol. Moreover, in GTA the accessibility of carbonyl oxygen to interact with shellac chains can be limited because of the existing methyl groups in the molecule (Qussi & Suess, 2006). Therefore, concentrations of GTA over 10% [w/w] were not miscible with shellac and had no further plasticizing effect on shellac films.

Addition of glycerol to shellac resulted in increased swelling rate of films at pH 1.2 as well as increased solubility at pH 7.5. However, the solubility of films at pH 6.8 was not affected (Figs. 2 and 4). The increased swelling rate of films at pH 1.2 might be explained by the competition between hydroxyl groups of glycerol and H^+ of the strongly acidic medium for reaction with ester groups of shellac. Due to some trans-esterification by glycerol, the rigid shellac matrix may be loosened, permitting the penetration of water and acid into the structure. This may explain the swelling observed under acidic conditions.

Addition of GTA to shellac resulted in a marginally increased swelling rate at pH 1.2 (Fig. 4a), which might be explained due to the limited interaction between the carbonyl oxygen atom of GTA with shellac chain. Although the solubility of films containing GTA at pH 6.8 was higher than that of the films containing glycerol (Fig. 2b), the better release profile of the probiotic microorganisms from the coatings containing glycerol in SIF (Tables 3 and 4) can also be explained by partial trans-esterification with formation of free carboxyl groups in the shellac molecules and gelation of coatings during exposure to SGF. The free carboxyl groups are responsible for increasing the solubility of coatings at alkaline pH values. In order to confirm this, in an unpublished study the solubility of films containing shellac and glycerol or shellac and GTA was investigated under physiological condition, this means after 2 h exposure to pH 1.2 and immediate transfer to pH 6.8 or 7.5 for 3 h. The solubility of films containing 5% [w/w] glycerol was increased at pH 6.8 or 7.5 from 8.5% to 20% [w/w] compared to exposure to pH 6.8 or 7.5 without prior exposure to pH 1.2.

5. Conclusion

Since probiotic microorganisms are highly sensitive to low gastric pH and given the limited number of approved excipients for the development of nutritional supplements, we have improved the enteric coating properties of shellac. Glycerol had the best plasticizing effect on shellac, resulting in the improved elasticity of films and increased solubility at pH 7.5. Addition of sodium alginate and PVP resulted in increased solubility of shellac films in simulated intestinal fluid. Using *E. faecium* M74 or *B. bifidum* 12 the formulations containing 5% [w/w] glycerol as well as formulations containing 5% [w/w] sodium alginate or 10% HPMC [w/w], or up to 20% [w/w] PVP showed the best enteric coating properties. In the case of *L. reuteri* ATCC 55730, the addition of 5% [w/w] sodium alginate to shellac did not protect the microorganisms against the acidic pH of the SGF.

Furthermore, these formulations maintained sufficient cell stability during 4 months of storage at 5 °C.

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