European Journal of Pharmaceutics and Biopharmaceutics 139 (2019) 240–245

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

# Ultra–fast disintegrating ODTs comprising viable probiotic bacteria and HPMC as a mucoadhesive

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ARTICLE INFO

*Keywords:*
Mucoadhesion
Mucoadhesion testing
Buccal mucosa
Orodispersible tablets
HPMC
Lactobacilli
Disintegration time
Granulation

ABSTRACT

Orodispersible tablets (ODTs) are a convenient dosage form and a recent trend in formulation development. The fast disintegration is accompanied by rapid removal of the active principle and the excipients from the mouth due to saliva flow and swallowing. Probiotic bacteria are a promising strategy to fight disease with bacterial aetiology in the mouth, but a certain residence time in the oral cavity is inevitable to exert their positive effects. The addition of a mucoadhesive polymer, like hydroxypropyl methylcellulose (HPMC), is an auspicious strategy to prolong this residence time. Nevertheless, the disintegration time of the tablets should still meet the acceptance level from the FDA (< 30 s). To reach intimate contact of bacteria and mucoadhesive polymer on the one hand and to support fast disintegration on the other hand, granulation of probiotic bacteria and mucoadhesive HPMC with a methacrylic acid copolymer was performed first. Moreover, high mucoadhesion could be obtained because bacteria and mucoadhesive polymer could interact more strongly with the mucosa after the ODT disintegrated and the methacrylic acid copolymer dissolved in the pH neutral saliva.

## 1. Introduction

Probiotics are commonly defined as: “Live microorganisms which when administered in adequate amounts confer a health benefit on the host.” [1] Probiotics are traditionally applied to the gastrointestinal tract, but other fields of application are also coming into the focus, like the oral cavity. Oral diseases with a microbiological aetiology include plaque-associated diseases like dental caries, gingivitis and periodontitis, which are a potential target for probiotic treatment. Plaque-associated diseases and fungal infections, as well as oral malodour are in the focus of oral probiotic research. Several health effects have been shown for *Lactobacillus* species in the field of periodontal disease, like a reduction of periodontopathic bacteria [2,3], in particular *P. gingivalis* [4,5]. Moreover, co-aggregation with disease-associated strains, like *P. endodontalis, T. forsythia,* or *P. gingivalis*, can be another mode of action [5–7]. Periodontal disease come along with inflammation of periodontal tissue. Such inflammation might be reduced using a probiotic treatment [4], by decrease of pro-inflammatory cytokines, like TNF-α or IL-8 [8,9] or by reduction of nitrite/nitrate, prostaglandin E2 and matrix metalloproteinase [10]. Furthermore, the immune system can be positively influenced by probiotics [11–13], by enhancing the activity of natural killer cells [14]. Finally, also clinical effects, like a significant reduction of the gingival index [15,16] and plaque scores [16,17], were found by using a probiotic treatment. Dental caries is associated with *S. mutans*, but there are also hints towards an association with some lactobacilli species [18–20]. Contradictory, lactobacilli from caries-free people exert a more effective inhibition of mutans-streptococci than lactobacilli isolated from caries-active subjects in-vitro [21] and certain *Lactobacillus* strains even co-aggregate with selected oral streptococci [22]. Some studies have been published that hint towards an anti-caries effect of lactobacilli [23–26]. By now the role of lactobacilli in caries is not absolutely clarified, but it is probably more associative than causative.

For the administration of probiotics to the patient, an adequate dosage form ensuring on the one hand storage stability of the active principle and on the other hand high patient adherence is mandatory. ODTs have the typical advantages of solid dosage forms including accurate dosing, high stability and easy manufacturing with means of standard equipment for tablet production. Moreover, orodispersible tablets disintegrate rapidly in the mouth, without the need for additional water for intake, because the tablets disintegrate due to the remaining saliva available in the mouth. Hence, it is not necessary to suck an ODT for several minutes like a lozenge. It is an appropriate dosage form for patients having difficulties with swallowing tablets or hard gelatin capsules, which occurs in 1 to 2 out of 4 patients [27,28]. The auspicious advantages of orodispersible tablets led to increased interest

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https://doi.org/10.1016/j.ejpb.2019.03.022
Received 22 October 2018; Received in revised form 20 February 2019; Accepted 30 March 2019
Available online 01 April 2019
0939-6411/ 

and a remarkable number of 124 marketed products in the European market, as reported by Pinho et al. [29]. After administration of a probiotic ODT, the probiotic bacteria are instantaneously released at the side of action. However, the expected residence time in oral cavity is limited, because the probiotics are removed by constant flushing with saliva, with up to 7 ml/min [30], apart from eating and drinking. A formulation strategy to overcome these challenges when delivering live probiotic bacteria to the oral cavity needs to be developed. An innovative approach is the inclusion of mucoadhesive polymers into an ODT formulation to prolong the residence time in the oral cavity. A large variety of mucoadhesive polymers is described in literature. For this study HPMC was chosen, because it is frequently used [31–35] and it is compatible with lactobacilli as ascertained in a preliminary study (data not shown here). Moreover, ionic interactions with other excipients can be neglected, because of its neutral character. It is a semi-synthetic polymer with a cellulose backbone which is characterised by moderate mucoadhesive properties. The mechanism of mucoadhesion for such a neutral polymer is mainly diffusion and interpenetration into the mucus layer [36]. Moreover, hydrogen bonding could also slightly contribute to mucoadhesion [37]. At the same time, HPMC is used for gel formation or as ingredient in matrix formulations. This might be contradictory to its use in ODTs because it can be expected, that the addition of HPMC would increase tablet disintegration due to the formation of a viscous gel that might hinder further penetration of water into the tablet [38]. This phenomenon has already been described for sublingual tablets, where increasing amounts of HPMC in the formulation led to increased disintegration time [39]. Hence, a strategy is required which allows for both mucoadhesion and fast disintegration within less than 30 s [40]. A granulation procedure of probiotics together with mucoadhesive polymer might improve tablet disintegration. Moreover, it might be a promising strategy to reach intimate contact between the probiotics and the mucoadhesive polymer. Wet massing with an organic solution of a suitable polymer as high shear process was chosen as granulation process. By using an organic solvent, the drying process after granulation can be performed at low temperature and the final product has a low water activity which is known to be beneficial for the storage stability of probiotic bacteria [41,42]. In case of dry granulation, the drying process would be overall omitted, but the compression during compaction on the one hand and during tablet production on the other hand might lead to lower bacterial counts as consequence of higher compression forces [43]. As a suitable binder polymer for granulation Eudragit® L 100-55, an anionic copolymer based on methacrylic acid and ethyl acrylate (1:1), was selected, which is soluble in isopropyl alcohol. Eudragit® L 100-55 is insoluble at low pH, e.g. gastric fluid, due to protonation of the acidic groups. At a pH above 5.5, like in the oral cavity, it dissolves, due to salt formation of the methacrylic acid moieties in the polymer [44] and the probiotics and mucoadhesive polymer can extensively interact with mucosa.

The aim of this study was to develop a probiotic orodispersible tablet with ultra-fast disintegration. The FDA states an acceptance level of maximum 30 s for the disintegration time of orodispersible tablets [40]. To maintain ultra-fast disintegrating ODTs we defined a target value with 20 s as mean value. The formulation should contain a mucoadhesive polymer like HPMC to attain sustained probiotic delivery to the oral mucosa while retaining ultra-fast tablet disintegration. To this end the suitability of a granulation process of viable probiotic material plus mucoadhesive excipient needs to be investigated, as well as the effect on disintegration time and mucoadhesion of the ODTs.

## 2. Material and methods

### 2.1. Material

*Lactobacillus plantarum* 299v (Lp299v) and *Lactobacillus paracasei* 8700:2 (Lp8700:2) were kindly supplied by Probi AB (Lund, Sweden). Lp299v powder was sieved through a 200 µm sieve, to avoid a sandy feeling in the mouth, but this was not necessary for fine Lp8700:2 material. Syloid® AL1–FP and Syloid® 244 FP were provided by Grace GmbH & Co. KG (Worms, Germany), Pruv® by JRS Pharma GmbH & Co. KG (Rosenberg, Germany), Kollidon® CL–SF by BASF SE (Ludwigshafen, Germany), Avicel® PH–112 by FMC BioPolymer (Philadelphia, USA), Pearlitol® 100 SD by Roquette frères (Lestrem, France) and Optamint® Lemon-Lime SD by Symrise AG (Holzminden, Germany). Metolose® 65SH50 was a gift from Shin-Etsu Chemical Co. (Tokyo, Japan). Potassium chloride from VWR International GmbH (Leuven, Belgium), sodium chloride from Caesar & Loretz GmbH (Hilden, Germany), sodium hydroxide from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Eudragit® L 100-55 from Evonik Industries AG (Darmstadt, Germany) were Ph.Eur. grade. Hydrochloric acid, α-Amylase from *Bacillus subtilis* and mucin from porcine stomach type III were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Tween™ 80 from Croda GmbH (Nettetal, Germany) and di-potassium hydrogen phosphate trihydrate from Merck KGaA (Darmstadt, Germany). MRS-agar and peptone were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Anaerogen™ from Thermo Fisher Scientific Inc. (Waltham, USA), Anaerocult® from Merck KGaA (Darmstadt, Germany), nitrogen 5.0 from Westfalen AG (Muenster, Germany) and isopropyl alcohol from Brenntag (Muelheim an der Ruhr, Germany).

### 2.2. Granulation process

Granulation of Lp299v with or without HPMC was performed using a Somakon LabMixer (Somakon Verfahrenstechnik UG, Luenen, Germany) with a 0.5 l vessel at 270–300 rpm. The vessel was continuously purged with nitrogen gas. The granulation fluid, 12.5 % (w/w) Eudragit® L 100–55 solution in isopropyl alcohol, was added dropwise using a syringe and the rotation speed was increased during the process up to 400 rpm to enable sufficient mixing. At the end of the process, the scrapper (level II) was additionally turned on. The wet-massed material was sieved through a 710 µm sieve and dried in a desiccator, which was additionally purged with dehumidified compressed air to support drying. After drying (water activity $< 0.1$), the granules were finally sieved through a 500 µm sieve. The granulation procedure with Lp8700:2 was similar, but discontinuous flushing of nitrogen was performed, and a net was applied on the lid of the mixing vessel to reduce powder loss of the fine probiotic material. The overall composition of the probiotic granules is shown in Table 1.

### 2.3. Tablet production

In preliminary studies an optimized orodispersible tablet formulation for probiotic bacteria had been developed and the overall

**Table 1**
Composition [%] of probiotic granules.

| | Lp299v without HPMC granulated | Lp299v with HPMC granulated | Lp8700:2 without HPMC granulated | Lp8700:2 with HPMC granulated |
|---|---|---|---|---|
| Probiotic bacteria | 96.64 | 75.43 | 96.62 | 75.43 |
| HPMC | 0 | 21.42 | 0 | 21.42 |
| Eudragit® L 100-55 | 3.36 | 3.15 | 3.38 | 3.15 |

**Table 2**
Composition [%] of probiotic ODT formulations.

| | Lp299v or Lp8700:2 without HPMC ungranulated | Lp299v or Lp8700:2 without HPMC granulated | Lp299v or Lp8700:2 with HPMC ungranulated | Lp299v or Lp8700:2 with HPMC granulated |
|---|---|---|---|---|
| Probiotic material | 17.5 | 17.5[1] | 17.5 | 17.5[1] |
| HPMC | 0 | 0 | 5.0 | 5.0[1] |
| Eudragit® L 100-55 | 0 | 0.61[1] | 0 | 0.73[1] |
| Pearlitol® 100 SD | 15.75 | 15.75 | 15.75 | 15.75 |
| Optamint® Lemon-Lime SD | 0.5 | 0.5 | 0.5 | 0.5 |
| Avicel® PH-112 | 50.75 | 50.14 | 45.75 | 45.02 |
| Kollidon® CL-SF | 5.0 | 5.0 | 5.0 | 5.0 |
| Pruv® | 0.5 | 0.5 | 0.5 | 0.5 |
| Syloid® 244 FP | 5.0 | 5.0 | 5.0 | 5.0 |
| Syloid® AL1-FP | 5.0 | 5.0 | 5.0 | 5.0 |

[1] Intra-granular.

composition is outlined in Table 2.

The excipients were sieved through a mesh size of 315 µm except for Kollidon® CL–SF and Pearlitol® 100 SD. Firstly, bacteria or probiotic granules plus Pearlitol® 100 SD (filling agent and non-cariogenic sweetener) and Optamint® Lemon-Lime SD (flavour) were mixed for 5 min. Secondly, Avicel® PH-112 (binder) and Kollidon® CL–SF (super disintegrant) were added and mixed for 10 min. Finally, Pruv® (lubricant) and Syloid® AL1–FP (moisture protection) plus Syloid® 244 FP (glidant) were enclosed and final mixing was performed for 5 min. If a premixture of Pearlitol® 100 SD, Optamint® Lemon-Lime SD, Avicel® PH–112, Kollidon® CL–SF, Syloid® AL1-FP and Syloid® 244 FP had already been prepared, only the missing amounts and substances were included, and the total mixing time was reduced to 10 min. The formulations with ungranulated HPMC were prepared by initial mixing of probiotics and HPMC for 10 min to attain close contact. All the other excipients (except glidant and lubricant) were added and mixed for 10 min. Finally, Pruv®, Syloid® 244 FP and AL1–FP were enclosed and mixing was performed for another 5 min. A Turbula® T2C mixer (Willy Bachofen, Basel, Switzerland) was used for all mixing steps (30 rpm). The closely sealed mixing vessel was equipped with a desiccant bag to avoid uptake of water vapour. The batch size was 80 g and always yielded 17.5 % pure bacteria and 5.0 % HPMC as mucoadhesive polymer.

The preparation of the formulation was done in a dehumidified production environment to yield tablets with low water activity and to maintain viability of the probiotic bacteria. A single punch tablet press Korsch EK II (Korsch Pressen, Berlin, Germany) equipped with 10 mm round flat face tooling was used to produce tablets (200 ± 15 mg) with comparable hardness (40–55 N). The compression area of the tablet machine was encapsulated and ventilated by dehumidified compressed air to ensure a relative humidity below 15 % [45]. The water activity of the produced tablets was very low (< 0.1) and they were stored in HD-PE tablet jars with a desiccant bag.

### 2.4. Crushing strength

The test was performed according to Ph.Eur. 2.9.8. and the measurement was carried out on 10 tablets of each batch using a hardness tester PTB111 (Pharma Test Apparatebau AG, Hainburg, Germany).

### 2.5. Disintegration time

The disintegration time of six tablets was determined according to Ph.Eur. using a disintegration tester Erweka ZT3 (Erweka GmbH, Heusenstamm, Germany). Only one tablet was measured at a time to determine the time for disintegration precisely. The temperature of the water was 37 ± 2 °C and the disintegration fluid was changed completely after measuring 3 tablets.

### 2.6. Survival rate

An amount of 0.1–1.0 g probiotic powder or probiotic granules was soaked in diluent (8.5 g/l NaCl, 1.0 g/l peptone) to reach a total mass of 100.0 g and was homogenised by means of a magnetic stirrer for 15 to 20 min. A serial dilution was performed under a laminar air flow bench using Dilushaker III digital (LabRobot Products AB, Stenungsund, Sweden) with Dilucups including MRD medium. A volume of 100 µl of the last 2 to 3 dilutions was pipetted onto MRS-agar plates and distributed using a sterile bent rod. The samples were incubated at 37 ± 1 °C for 2–5 d under anaerobic conditions (Anaerogen™). Plates with 25 up to 350 colonies were counted and based on this number the viable count was calculated. If only one plate contained the specified amount of bacteria, the viable count was calculated as follows:

$$Viable\ \ count\,[CFU/g] = \frac{Number\ \ of\ \ colonies * 10^{dilution}}{m_{sample}(g) * 0.1} \quad (1)$$

If 2 plates were between 25 and 350 colonies:

$$Viable\ \ count\,[CFU/g] = \frac{\frac{Summary\ \ of\ \ number\ \ of\ \ colonies}{1,1} * 10^{lowest\ \ dilution}}{m_{sample}(g) * 0.1} \quad (2)$$

The measurements were at least performed in duplicate and the survival rate was calculated on basis of the amount and the viable count of pure probiotics before and after the granulation process.

### 2.7. Mucoadhesion

Mucoadhesion was tested according to Hoffmann and Daniels [46]. In brief the method was as follows.

#### 2.7.1. Preparation of porcine buccal tissues

The buccal tissue was isolated directly after the death of the pigs and frozen at −28 °C. The final preparation of the tissue was performed immediately before the experiment. The final mucosa was 25 mm in diameter, 2 ± 1 mm thick and was fixed in a metal cell for better handling.

#### 2.7.2. Determination of mucoadhesion

The mucosa was wetted with 2–3 drops of artificial saliva and equilibrated for 15 min in a mucoadhesion cell, which was placed in a water bath Memmert W200 (Memmert Gmbh & Co. KG, Schwabach, Germany) at 37 °C and thereafter again wetted with 2 drops of saliva. 50 ± 1 mg of the formulation, which is equal to a quarter of the tablet, was used for testing. After placing the tablet on the mucosa, it was flushed with 0.5 ml/min artificial saliva for 60 min by means of a HPLC pump Model 510 (Waters GmbH, Eschborn, Germany). The amount of probiotic bacteria, which remained on the mucosa and the amount,

which were flushed away during the test, were quantified by plate-counting method. The total recovery of probiotics was ≥ 80 %. Adhesion on the mucosa was expressed as percentage of the total bacterial count applied per tablet. All measurements were performed in triplicate.

### 2.8. Statistical analysis

All data were obtained by repeated measurements (n ≥ 3) and are represented as arithmetic mean ± standard deviation. The data of different samples were analysed by a one-way analysis of variance (ANOVA) with a significance level of 95 % ($p < 0.05$) and Student-Newman-Keuls test. Data pairs were analysed by t-Test ($p < 0.05$). Data that are significantly different ($p < 0.05$), are marked with an asterisk (*).

## 3. Results and discussion

### 3.1. Granulation of probiotics

The wet granulation procedure of pure probiotic bacteria with an isopropyl alcohol solution, based on Eudragit® L 100-55, did not affect the viability of the tested probiotics, despite applied shear stress and the use of an organic solvent. Both tested probiotic strains showed no decrease in cell numbers after the granulation process (Fig. 1). It was concluded that the mild granulation conditions with a short contact time of bacteria and pure organic solvent plus the mild drying conditions at room temperature are well tolerated by the lactobacilli.

Plain ODTs including granulated probiotics disintegrated significantly faster than those with ungranulated probiotic powder (Fig. 2). The disintegration time was significantly reduced two- or even threefold for both materials.

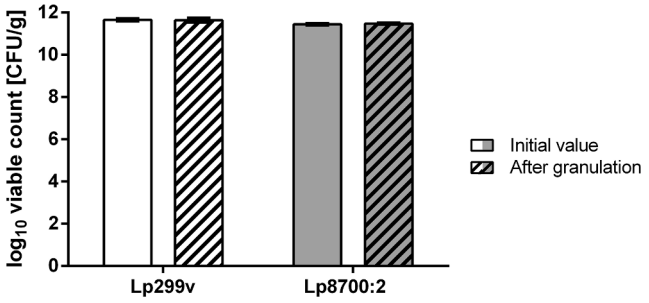


**Fig. 1.** Viable count of two probiotic strains before and after granulation using Eudragit® L 100-55 solution.

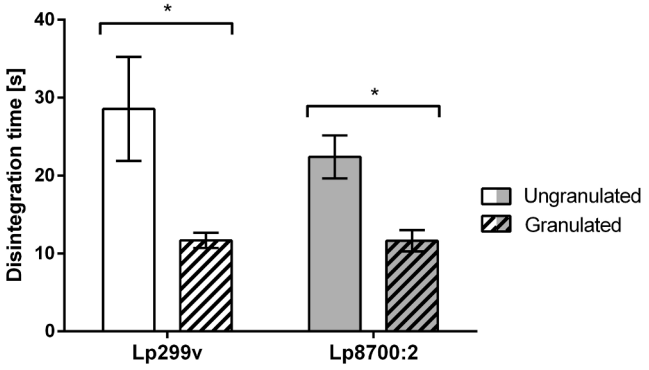


**Fig. 2.** Influence of granulation of two different probiotic Lactobacillus strains on tablet disintegration.

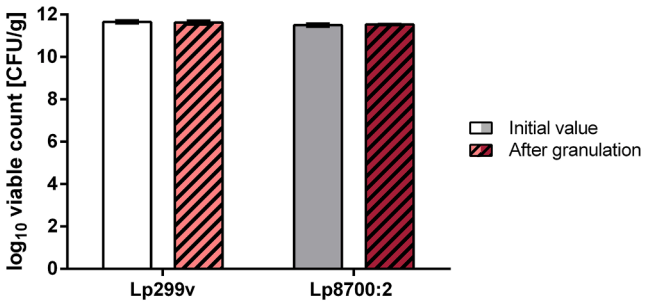


**Fig. 3.** Viable count of two probiotic strains before and after granulation with HPMC using Eudragit® L 100-55 solution.

**Table 3**
Disintegration time of ungranulated probiotic tablets with and without mucoadhesive HPMC.

| | Lp299v | Lp8700:2 |
|---|---|---|
| Without HPMC | 28.56 ± 6.67 s | 22.39 ± 2.77 s |
| With HPMC | 42.80 ± 3.48 s | 45.51 ± 8.25 s |

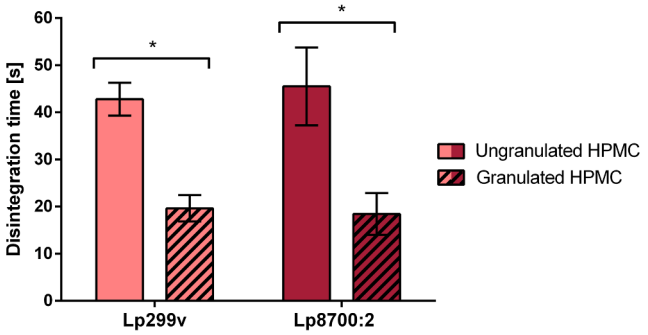


**Fig. 4.** Influence of granulation of two different probiotic Lactobacillus strains with HPMC on tablet disintegration.

### 3.2. Probiotic ODTs including mucoadhesive polymer

In order to achieve the desired retention of the probiotic bacteria in the buccal cavity, the ODTs were supplemented with HPMC as a mucoadhesive polymer. To this end, the probiotics and HPMC were mixed and wet massed with the enteric polymer. The intention of this was to bring the probiotics and the mucoadhesive polymer into immediate vicinity and to be still able to maintain ultra–fast disintegration of the tablets. The viability of the probiotics plus mucoadhesive polymer after granulation was measured and the obtained results are shown in Fig. 3. Obviously, the granulation procedure after addition of HPMC did not affect the viability of the bacteria negatively.

As HPMC has a prolonging effect on the disintegration, the maximum amount of HPMC, that could be added to the formulation without exceeding 20 s a mean disintegration time (ultra–fast disintegration) was determined in a preliminary study to be 5.0 %. As expected, the addition of HPMC led to an increase of the disintegration time compared to the ungranulated formulations without any mucoadhesive polymer (Table 3).

Nevertheless, prior granulation of probiotics plus HPMC again showed a positive effect on tablet disintegration. As demonstrated in Fig. 4, the disintegration time was significantly reduced and ultra-fast disintegration (< 20 s) was accomplished using the granulation method. The overall results of Lp299v and Lp8700:2 were comparable.

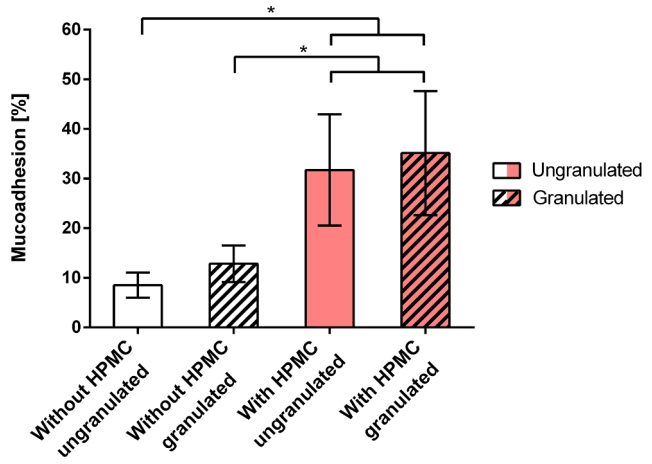


**Fig. 5.** Influence of different formulation concepts on the mucoadhesion of Lp299v to buccal porcine mucosa after 60 min flushing with 0.5 ml/min artificial saliva when applied in different tablet formulations.

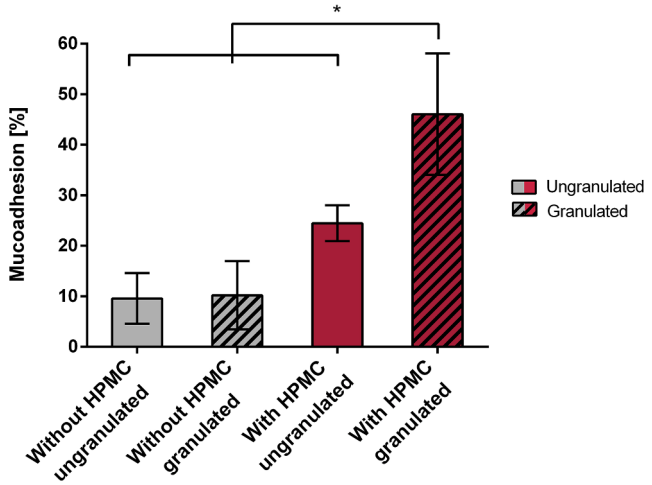


**Fig. 6.** Influence of different formulation concepts on the mucoadhesion of Lp8700:2 to buccal porcine mucosa after 60 min flushing with 0.5 ml/min artificial saliva when applied in different tablet formulations.

### *3.3. Mucoadhesion*

Furthermore, the effect of HPMC, incorporated into the probiotic granules, on the mucoadhesion performance was investigated. For Lp299v the results were as follows: from the pure ungranulated probiotics only 8.5 ± 2.5 % remained on the mucosa (Fig. 5). This value was comparable to granulated probiotics without any mucoadhesive polymer. Hence, the granulation of pure probiotics did not influence mucoadhesion. The addition of HPMC led to a significant increase in mucoadhesion, but as shown in Table 3, also to an increase in disintegration time. By using probiotic granules with HPMC, the number of bacteria adhering to the mucosa was raised to 35.1 ± 12.5 % and significantly increased, despite maintaining fast disintegration of the tablets.

The results for Lp8700:2 were similar to those of Lp299v. Without addition of a mucoadhesive polymer approximately 10 % of Lp8700:2 adhered after 60 min flushing, whether granulated or not (Fig. 6). The addition of HPMC assisted adhesion to the mucosa. The use of HPMC incorporated into probiotic granules resulted in significantly higher mucoadhesion compared to the ungranulated polymer. The intimate contact of Lp8700:2 and HPMC in the granules facilitated mucoadhesion of the bacteria. Therefore, granulation was a successful strategy to fasten tablet disintegration, and ensure increased mucoadhesion.

## 4. Conclusion

This study revealed that it is possible to formulate live probiotic bacteria in fast disintegrating orodispersible tablets. However, some of the probiotic formulations do not fulfil the FDA's acceptance limit for the disintegration time of ODTs. This could be overcome by granulating the bacteria with an enteric polymer. If the wet massing was done with 2-propanol as solvent, the viability of the bacteria was not impaired. A prolonged mucoadhesion could be achieved by adding HPMC as a mucoadhesive polymer. Again, granulation with methacrylic acid copolymer could cope the negative influence of HPMC on the disintegration time while maintaining the viability of the probiotics and ultra-fast disintegrating tablets could be prepared. With Lp299v a slight trend and with Lp8700:2 a significant improvement of the amount of adhering microorganisms could be observed after the granulation step using HPMC. This implies that the intimate contact of HPMC and the probiotic bacteria favours their attachment to the buccal mucosa. Furthermore, the mucoadhesion tests proved that the enteric polymer is dissolved rapidly enough in the artificial saliva to allow the HPMC to develop its mucoadhesive properties.

## Acknowledgement

The authors would like to thank Grace GmbH & Co. KG (Worms, Germany) for the provision of Syloid® products, JRS Pharma GmbH & Co. KG (Rosenberg, Germany) for donation of Pruv®, Roquette frères (Lestrem, France) for supplying Pearlitol® 100 SD and IMCD Deutschland GmbH & Co. KG (Cologne, Germany) for donating Avicel® PH-112. Metolose® 65SH50 as a gift from Shin-Etsu Chemical Co. (Tokyo, Japan). Flavour was provided by Symrise AG (Holzminden, Germany) and probiotics by Probi AB (Lund, Sweden).

## Statement of ethics

Porcine buccal mucosa was received from the department of experimental medicine of the University Hospital Tuebingen, where the pigs were sacrificed in the course of other experiments. Buccal tissue was isolated directly after the death of the animals. The experiments had been approved by the ethics committee of the University Hospital Tuebingen. Moreover, porcine buccal mucosa was isolated immediately after the pig's slaughter at a local butcher (Grießhaber, Oeschingen, Germany). The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tuebingen (registration number: DE 08 416 1052 21).

## Disclosure statement

The project was financially supported by Symrise AG, but with no impact on the collection, analysis and interpretation of data or in writing of the report. Anja Hoffmann reports personal fees from Symrise AG and Rolf Daniels reports Grants from Symrise AG. In addition, Rolf Daniels and Anja Hoffmann have a patent EP 15 184849.6 pending.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.03.022.

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