Peer-reviewed version available at *Pharmaceutics* **2019**, *11*, 270; doi:10.3390/pharmaceutics11060270

Article

In vitro evaluation of Eudragit matrices for oral delivery of BCG vaccine to animals

Imran Saleem [1], Allan Coombes [2] and Mark Chambers [3,4,*]

[1] Liverpool John Moores University, School of Pharmacy and Biomolecular Sciences, Byrom Street, Liverpool, L3 3AF, United Kingdom; I.Saleem@ljmu.ac.uk
[2] University of Queensland, School of Pharmacy, Pharmacy Australia Centre of Excellence, 20 Cornwall Street, Woolloongaba, QLD 4102, Australia; allancoombes1@gmail.com
[3] Animal and Plant Health Agency, Department of Bacteriology, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom; mark.chambers@apha.gov.uk
[4] University of Surrey, School of Veterinary Medicine, VSM Building, Daphne Jackson Rd, Guildford GU2 7AL, United Kingdom; m.chambers@surrey.ac.uk

* Correspondence: mark.chambers@apha.gov.uk

Abstract: Bacillus Calmette-Guérin (BCG) vaccine is the only licensed vaccine against tuberculosis (TB) in humans and animals. It is most commonly administered parenterally but oral delivery is highly advantageous for immunisation of cattle and wildlife hosts of TB in particular. Since BCG is susceptible to inactivation in the gut, vaccine formulations were prepared from suspensions of Eudragit L100 copolymer powder and BCG in PBS, containing Tween 80, with and without the addition of mannitol or trehalose. Samples were frozen at -20°C, freeze-dried and the lyophilised powders were compressed to produce BCG-Eudragit matrices. Production of the dried powders resulted in a reduction in BCG viability. Substantial losses in viability occurred at the initial formulation stage and at the stage of powder compaction. Data indicated that the Eudragit matrix protected BCG against simulated gastric fluid (SGF). The matrices remained intact in SGF and dissolved completely in SIF within three hours. The inclusion of mannitol or trehalose in the matrix provided additional protection to BCG during freeze-drying. Control needs to be exercised over BCG aggregation, freeze-drying and powder compaction conditions to minimise physical damage of the bacterial cell wall and maximise the viability of oral BCG vaccines prepared by dry powder compaction.

Keywords: BCG; Eudragit, oral vaccine; tuberculosis; in vitro viability

1. Introduction

According to the World Health Organisation 2018 Global Tuberculosis (TB) Report, TB remains one of the top ten causes of death and the leading cause of morbidity from a single infectious agent worldwide [1]. Similarly, livestock TB constitutes a major animal health problem. It has been estimated that >50 million cattle are infected worldwide, costing US$3 billion annually due to reduced cattle productivity, culling and movement and trade restrictions [2]. In addition, the global health burden is increased by the fact that TB in animals is an important zoonosis, causing disease in humans, particularly through consumption of unpasteurised milk or by aerosol transfer from infected animals. This factor prompted the WHO and allied organisations to publish in 2017 a joint roadmap for tackling zoonotic TB [3].

The Bacillus Calmette-Guérin (BCG) is a live bacterial vaccine that has been used in humans since 1921. The vaccine was registered for intramuscular administration to badgers in the UK in 2010, but is not currently registered for use in domestic livestock. BCG has been evaluated in numerous trials and in many different species since its creation, and although efficacy can be variable, it is generally considered an effective vaccine when administered parenterally, reviewed in [4]. However, delivery of BCG to mucosal surfaces through oral or intranasal administration is highly desirable to

avoid the use of needles for humans and domesticated animals and to simplify immunisation of wildlife, particularly through presentation of BCG incorporated in bait [5].

The oral route is extensively used for drug delivery, even to exotic species [6] due to the ease of administration and cost effectiveness. Oral vaccines have also been widely investigated but the difficulty of protecting protein and peptide antigens against degradation in the harsh conditions of the gastrointestinal tract, notably the low pH and the presence of enzymes, remains a major obstacle to progress in this area [7-9]. For BCG, data point to the need to protect the live vaccine against degradation in the gut if it is to be effective when administered orally. For example, in experimental studies in humans doses of oral BCG were administered either with 2% sodium bicarbonate (to neutralise gastric acidity) immediately before [10] or simultaneously with vaccine [11]. More convincingly, intra-gastric administration of BCG to brush-tailed possums (the principal wildlife reservoir of TB in New Zealand) was less effective than vaccine administered by the same route in combination with a drug to reduce gastric acidity or when administered intra-duodenally [12,13]. Protection of BCG against degradation in the gut is also the basis of efforts to formulate BCG in a lipid matrix for oral delivery to wildlife [14-16].

Many pharmaceutical oral dosage forms are enteric coated with acid-resistant Eudragit® copolymers to protect the stomach mucosa against irritation by drugs including non-steroidal anti-inflammatory drugs (NSAIDS) or to prevent degradation of the active component on exposure to gastric acid or enzyme action [17,18]. The Eudragit® family of copolymers is based on anionic polymers of methacrylic acid and methacrylates. They contain –COOH as a functional group and dissolve at specific pH values between 5.5 and 7.0, depending upon the grade. Furthermore, the copolymers are available as powders, aqueous dispersions and in solution in inorganic solvents, which renders them highly versatile for formulation of drug delivery systems. Eudragit has previously been employed for encapsulation of protein antigens, including colonisation factor antigen I and F4 fimbriae antigen of *Escherichia coli* (ETEC) [19,20]; the latter being tested as an oral tablet presentation for piglets. Metabolically active lactic acid bacteria have been incorporated in enteric-coated tablets [21] and granules [22], and live attenuated Ty21a *Salmonella* typhi have been formulated as an oral vaccine in enteric-coated capsules [23]. Recently, a laminate presentation of *Bifidobacterium breve* bacteria dried onto Eudragit L100 film has been shown to protect the viable bacterial cells from inactivation by simulated gastric fluid (SGF) [24].

The aim of the present study was to evaluate the potential of a BCG-Eudragit matrix formulation for oral vaccination against TB, with a focus on potential incorporation into bait for delivery to badgers [5]. We used Eudragit L100 supplied in powdered form. The polymer dissolves at a pH above 6.0 and has been used as an enteric coating system for oral preparations relevant to this study [19,20,24]. The protective capacity of the matrix formulation towards BCG was evaluated by exposure of samples to simulated gastric and intestinal fluids.

2. Materials and Methods

2.1. Materials

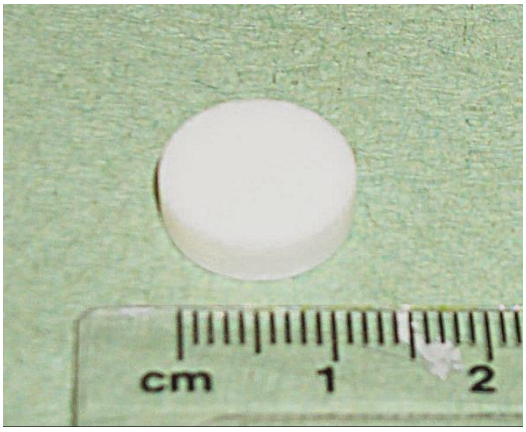
BCG Pasteur strain (obtained originally from the Statens Serum Institute, Copenhagen, Denmark) was grown in Middlebrook 7H9 liquid medium plus Albumin Dextrose Catalase (ADC) Supplement (Beckton & Dickinson, UK) to a concentration of approximately 10^8 colony forming units (cfu)/mL. Aliquots were prepared and stored at -80°C for subsequent experiments. Eudragit L100 polymer (ratio of free carboxyl groups to ester groups approximately 1:1, MW 13.5 kDa) was provided by Röhm GmbH (Darmstadt, Germany). Polyoxyethylene-sorbitan monooleate (Tween® 80), mannitol and trehalose were obtained from Sigma-Aldrich (Gillingham, UK).

2.2. Preparation of BCG-loaded Eudragit® matrices

Suspensions of Eudragit L100 powder in PBS (1.9mL, 18% w/v) were produced in glass vials with the addition of Tween® 80 (0.1 % w/v) (formulation A). Separate formulations contained, in addition, 0.2 % w/v mannitol (formulation B) or 0.2 % w/v trehalose (formulation C). The Eudragit® aqueous dispersions were acidic and the pH was adjusted to 7.0 using 1 M NaOH prior to sterilisation

by autoclaving at 15 psi, 121 °C for 15 minutes. BCG Pasteur from stock (100µL, approx. 10^8 cfu/mL) was added to the sterile suspension and retained at -20 °C for 6 h prior to freeze-drying (60 mbar (6,000 Pa) pressure, -50 °C for 24 h (n=3)) using an Edwards Modulyo freeze-drier (Edwards, Burgess Hill, UK). Following freeze-drying, powders were compressed using a Specac KBr disc compressor (Specac, Orpington, UK) by applying a 4 tons load for 3 minutes, to produce 13 mm diameter, 2.2 mm thickness BCG-loaded Eudragit matrices (Figure 1).

Figure 1. Freeze-dried powders were compressed to produce BCG-loaded Eudragit matrices of 13 mm diameter and 2.2 mm thickness.



2.3. BCG viability during matrix formulation

Samples of formulation A were analysed for BCG viability during each stage of BCG-Eudragit matrix production (formulation of suspensions, freezing at -20°C, freeze-drying and powder compaction). Samples of Formulations B and C were analysed for BCG viability during all stages, except after compaction. BCG was extracted from freeze-dried powders and solid matrices by resuspending in 10 ml PBS, pH 7.4 before dilution. Serial dilutions of resuspended samples were made in sterile water containing 0.05% Tween® 80. Aliquots (100 µL) of each dilution were spread onto Middlebrook 7H10 + Oleic ADC (OADC) agar plates (n=3, per dilution) and incubated at 37°C for 21 days. The number of cfu which resulted at each dilution was counted and converted to an average cfu.

2.4 BCG viability following matrix incubation in SGF and SIF

Individual BCG-Eudragit matrix samples (n=3) were placed in SGF (10 mL HCl, 0.1 M, pH 1.2) for 2 hours at 37°C in a water bath, removed and dried to constant weight at room temperature and then transferred to SIF (10 mL HEPES, pH 7.4) and retained at 37°C until they had fully dissolved. BCG was extracted from matrices exposed to SGF by dissolving the matrix in 10 mL HEPES solution (pH 7, 37 °C). Serial dilutions of BCG extracted from matrices incubated in SGF and SIF were made in sterile water containing 0.05% Tween® 80. Aliquots (100 µL) of each dilution were spread onto Middlebrook 7H10 + OADC agar plates (n=3, per dilution) and incubated at 37 °C for 21 days. The number of cfu which resulted at each dilution was counted and converted to an average cfu.

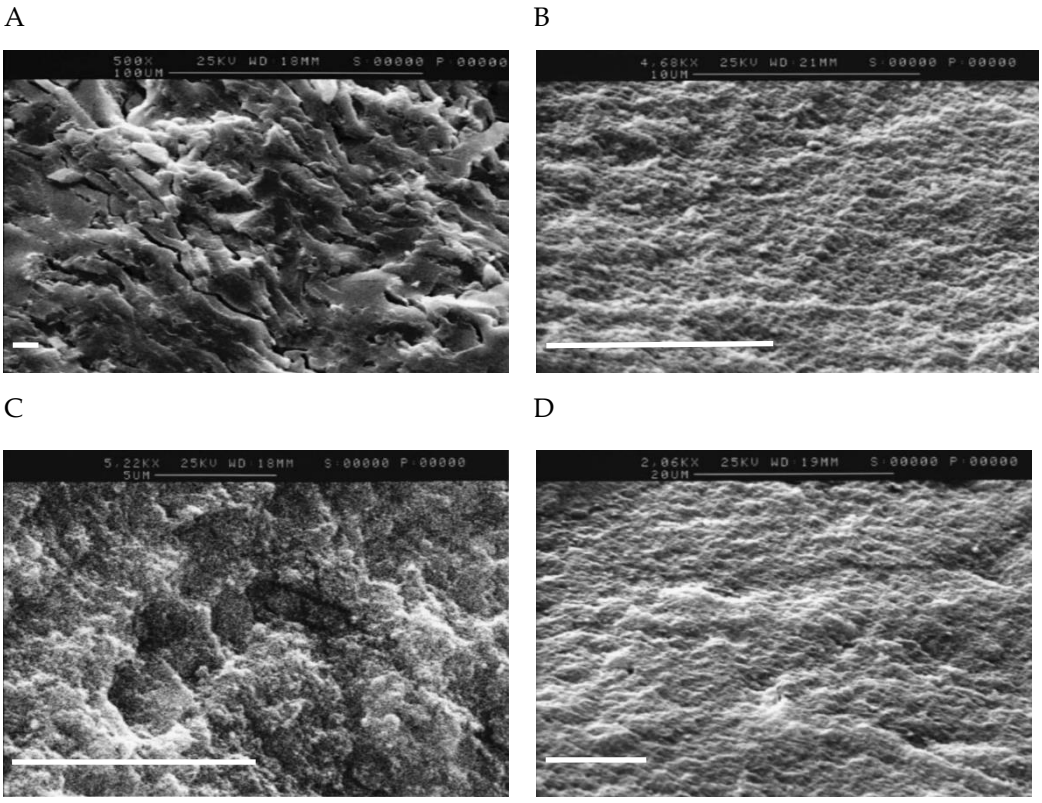
2.5. Statistical analysis

Analyses were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Losses in BCG viability after each stage of formulation were analysed by 2-way ANOVA with Tukey's multiple comparisons test against the $\log_{10}$ transformation of the raw data. The mean viability of BCG at each stage of evaluation was compared for differences between the three formulations using the multiple t test (each stage of evaluation being analysed separately to avoid assumptions about consistent standard deviations, using the raw data). Significant p values were identified using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Differences of $p < 0.05$ were considered significant throughout.

3. Results

The scanning electron micrographs in Figure 2 show the surface appearance of BCG-free matrices produced by compaction of lyophilised powders prepared from dispersions of Eudragit powder in PBS/Tween (formulation A) plus mannitol (formulation B) or trehalose (formulation C). Numerous pores and fissures are apparent in the lower magnification image of formulation A and at higher magnification for formulation C, compared with formulation B. The typical dense compact structure of BCG-free matrices formed by compaction of 'as received' Eudragit L100 powder alone is shown in Figure 2D. A more 'open' morphology would be expected to facilitate penetration of the BCG-loaded Eudragit matrices by gastric fluids and deactivation of exposed BCG. However, the presence of PBS salts in the lyophilised powders was considered advantageous, since it provided the possibility of local pH control within the porous matrices during transport through the stomach. Formulation A BCG-Eudragit matrices retained an average of 88 % of their initial weight after incubation in SGF for 2h (216.3 ± SD 8.2 mg reduced to 190.6 ± SD 4.4 mg). When these matrix samples were subsequently transferred from SGF to SIF, complete dissolution occurred within 2.3 h. Formulations B and C were not assessed in this way.

Figure 2. Scanning electron micrographs showing the surface appearance of Eudragit matrices produced by compaction of lyophilised powders prepared from a dispersion of Eudragit powder in: (**a**) PBS/Tween; (**b**) plus mannitol; (**c**) or trehalose; (**d**) or produced by compaction of 'as received' Eudragit L100 powder alone. Size-bar represents 10 μm.



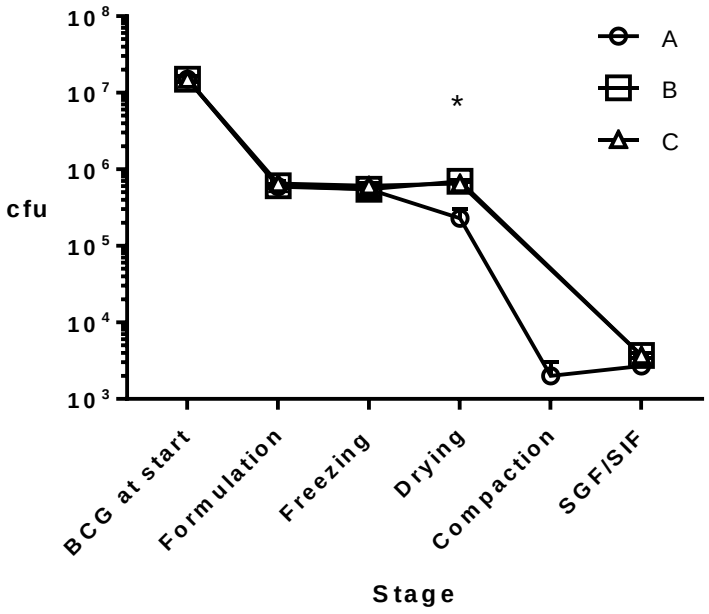
The concentration of the BCG stock solution after thawing was 1.48 x 10^8 (SD, 0.14 x 10^8) cfu/mL and 100μL was incorporated in each of the three types of matrix formulation. Table 1 shows the impact on BCG viability caused by matrix formulation (freezing of BCG/Eudragit co-suspensions, freeze-drying, dry powder compaction), and after exposure of BCG-Eudragit matrices to SGF and SIF. Figure 3 expresses the same data but illustrates the cumulative reduction in BCG viability for each formulation.

Table 1. Viability of BCG (cfu) at each stage of matrix production (data represent mean ± SD, n=3). For each matrix, 100 µL of BCG stock (1.48 ± 0.14 x 10^8 cfu/mL) was used, representing a starting BCG quantity of 1.5 x 10^7 cfu.

Matrix[1]	Suspension (pH 7)	Freezing (-20°C/6 h)	Freeze drying (24 h)	Dry powder compaction	Matrix exposure to SGF & SIF
A	5.9 ± 0.4 x10^5	5.5 ± 0.4 x10^5	2.3 ± 0.7 x10^5	2.0 ± 1.0 x10^3	2.7 ± 0.1 x10^3
B	6.1 ± 0.4 x10^5	5.5 ± 1.1 x10^5	7.0 ± 0.2 x10^5 *	ND	3.7 ± 0.3 x10^3
C	6.5 ± 0.5 x10^5	6.1 ± 0.5 x10^5	6.5 ± 0.3 x10^5 *	ND	3.7 ± 0.2 x10^3

[1] BCG-Eudragit matrix composition: A, Eudragit L100/2ml PBS /0.9ml Tween® 80 (0.1 % w/v); B, as A plus mannitol (0.2 %w/v); C, as A plus trehalose (0.2 % w/v). * P < 0.05 for pair-wise comparisons against matrix A.

Figure 3. Cumulative reduction in BCG viability (cfu) for each matrix formulation (A, B, C) through each stage. BCG-Eudragit matrix composition: (**a**) Eudragit L100/2ml PBS/0.9ml Tween® 80 (0.1 % w/v); (**b**) as (**a**) plus mannitol (0.2 %w/v); (**c**) as (**a**) plus trehalose (0.2 % w/v). * P < 0.05 for pair-wise comparisons of matrices B and C against matrix A.



According to 2-way ANOVA analysis of the log_{10}-transformed data, significant losses in BCG viability were apparently first encountered during the BCG/Eudragit powder suspension stage for all formulations, with reductions of 1.36 to 1.40 log_{10} (Table 2). No significant additional reduction in viability was caused by freezing the suspensions at -20°C for 6 h. Further reduction in BCG viability was encountered after freeze-drying matrix A, but not for matrices B and C, which included mannitol and trehalose, respectively. Thus, on completion of the freeze-drying stage there was significant retention of BCG viability for matrices B and C compared with A (Table 1, Figure 3). Freeze-dried formulations were compressed to form matrices and then incubated in SGF, followed by SIF. All formulations converged on an incremental loss of BCG viability to around 3.4 x 10^3 cfu (Table 1),

representing a total cumulative average loss in BCG viability of 3.65 $\log_{10}$ (range, 3.61 – 3.74 $\log_{10}$). For formulation A, we additionally assessed the impact of compaction on BCG viability. Compaction alone caused a significant, 100-fold reduction in apparent BCG viability compared with the viable concentration of BCG after freeze-drying (Table 2). It is noteworthy for this formulation that exposure of BCG-Eudragit matrices to SGF/SIF caused no further reduction in viability, despite their apparently porous morphology (Figure 2), indicating the protective effect of this matrix against low pH conditions. Although we did not assess the effect on BCG of compaction of the formulations containing mannitol (B) or trehalose (C), if we assume the impact of powder compaction on these formulations was equivalent to formulation A, then BCG viability in these matrices decreased little more after exposure to SGF and dissolution in SIF.

Table 2. Incremental changes in the viability of BCG ($\log_{10}$) incurred at each stage of matrix production (based on Table 1). The starting BCG concentration was 1.48 ± 0.14 x 10^8 cfu/mL, representing a starting BCG quantity of 1.48 x 10^7 cfu; equivalent to 7.17 $\log_{10}$.

Eudragit matrix[1]	Suspension (pH 7)	Freezing (-20°C/6 h)	Drying (24 h)	Compaction	SGF & SIF	Total
A	-1.40 *	-0.03	-0.38 *	-2.06 *	+0.13	-3.74
B	-1.38 *	-0.04	+0.10	ND	-2.28 *	-3.61
C	-1.36 *	-0.03	+0.03	ND	-2.24 *	-3.61

[1] BCG-Eudragit matrix composition: A, Eudragit L100/2ml PBS /0.9ml Tween® 80 (0.1 % w/v); B, as A plus mannitol (0.2 %w/v); C, as A plus trehalose (0.2 % w/v). * $P < 0.05$ for comparison against preceding column, e.g. for matrix A there is a significant reduction in viability between compaction and drying, and for all formulations between suspension and starting concentration.

4. Discussion

BCG-containing Eudragit matrices were formulated using a dry powder compaction approach in an effort to produce an oral BCG vaccine that retains high viability during transit through the gut. The matrices were physically stable at low pH (in SGF) and the data indicate that at least the matrix produced from PBS/Tween did not appreciably permit the low pH medium (SGF) to penetrate, causing major loss in BCG viability. Although we cannot say with certainty this was the case for the matrices containing mannitol or trehalose, it seems probable. Despite this, we encountered a total cumulative average loss in apparent BCG viability from start to finish of 3.65 $\log_{10}$. Analysis of the data show that significant losses occurred at the initial formulation stage and at the stage of compaction.

BCG has a tendency to aggregate [25], leading to an apparent effect of reducing the cfu count and being interpreted as a loss in viability. For this reason, all BCG-Eudragit matrix formulations included Tween 80, which is commonly added to BCG suspensions prior to culture or vaccine formulation to prevent aggregation [26-28]. Despite this, we think aggregation was the most likely explanation for the apparent loss in BCG viability during the initial formulation stage; indeed, aggregates of BCG were seen in the initial formulations under the light microscope (data not shown). Bacterial death at this point seems unlikely since the suspensions were adjusted to pH 7.0 before adding BCG.

A decided disadvantage of the dry powder compaction approach described here for production of oral BCG vaccine relates to the reduction of BCG viability following powder compaction (evident for formulation A). The application of pressure (e.g. French Press) is recognised as a highly efficient

way of disrupting the cell wall of mycobacteria [29]. The pressure normally applied (approx. 100 MPa) is around three times lower than the pressure used to produce the BCG-Eudragit matrices (290 MPa) in the present study. By contrast, a pressure of 5 MPa did not reduce the viability of live bacteria in tablets produced from hydroxypropyl methylcellulose acetate succinate (HPMCAS) [21]. Thus, the approach of formulating live BCG vaccines by dry powder compaction appears to be severely limited by the sensitivity of the bacteria to compaction. Attempts to reduce the duration and pressure of compaction resulted in matrices that were unstable in SGF (data not shown), suggesting that the inclusion of a binder (e.g. HPMCAS) would be of benefit in future formulations.

Gheorghiu et al [27] reported that freeze-drying results in loss of viability of BCG Pasteur and this was also our observation for matrix A. Loss in BCG viability during freezing due to ice crystal formation and/or salt crystal growth (arising from the use of PBS as the suspension medium) may have contributed to rupture of the bacteria cell wall. The addition of mannitol or trehalose provided protection to BCG during freeze-drying as expected from the literature. Mannitol is widely used as a cryo-protectant to improve the viability of lyophilised formulations and has been considered for the formulation of new, recombinant BCG vaccines for TB [30]. Similarly, the inclusion of trehalose is a common means of stabilising biomacromolecules during drying [31].

The key finding from this study is that formulation of BCG-Eudragit L100 matrices using dry powder compaction resulted in a significant reduction in viability from the start of formulation to final exposure to SGF/SIF, regardless of the formulation used. Although the minimum efficacious dose for oral BCG has not been determined for any species, typical efficacious doses in experimental studies in humans [11] and animals (reviewed in [4]) exceed 10^7 cfu. It therefore seems unlikely that the present approach of dry powder compaction would produce an efficacious oral vaccine. Nevertheless, the extensive application of Eudragit® for oral drug delivery recommends further studies to exploit the advantages of the material for production of oral BCG vaccines. Possible strategies include the incorporation of a binder with the dry powders to reduce the compaction forces required for matrix production and thus minimise physical damage of the bacterial cell walls and sonication to disperse aggregates of BCG before freezing.

5. Conclusions

BCG-Eudragit L100 matrices were produced by dry powder compaction to investigate their potential as oral vaccines for animals. Production of BCG-Eudragit dry powders resulted in significant loss of apparent viability of BCG. Matrix production by powder compaction resulted in a further reduction in BCG viability of around 100 fold. However, incubation of at least one of the matrices in SGF followed by SIF showed no further reduction in viability, indicating the capacity of the matrices to control SGF ingress and protect the residual live bacteria.

Author Contributions: conceptualization, M.C. and A.C.; methodology, A.C. and I.S.; formal analysis, M.C., A.C. and I.S.; investigation, I.S.; resources, M.C.; data curation, I.S. and M.C.; writing—original draft preparation, I.S.; writing—review and editing, M.C., A.C. and I.S.; visualization, M.C.; supervision, A.C. and M.C.; project administration, M.C.; funding acquisition, M.C.

Funding: This research was funded by Department for Environment, Food and Rural Affairs, UK, grant number SE3246.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, but were consulted in the decision to publish the results.

References

1. World Health Organization. *Global Tuberculosis Report 2018*; World Health Organization: 2018.
2. Waters, W.R.; Palmer, M.V.; Buddle, B.M.; Vordermeier, H.M. Bovine tuberculosis vaccine research: historical perspectives and recent advances. *Vaccine* **2012**, *30*, 2611-2622, doi:10.1016/j.vaccine.2012.02.018.
3. World Health Organization. *Roadmap for zoonotic tuberculosis*; World Health Organization: 2017.
4. Buddle, B.M.; Vordermeier, H.M.; Chambers, M.A.; de Klerk-Lorist, L.M. Efficacy and Safety of BCG Vaccine for Control of Tuberculosis in Domestic Livestock and Wildlife. *Front. Vet. Sci.* **2018**, *5*, Article 259.
5. Gowtage, S.; Williams, G.A.; Henderson, R.; Aylett, P.; MacMorran, D.; Palmer, S.; Robertson, A.; Lesellier, S.; Carter, S.P.; Chambers, M.A. Testing of a palatable bait and compatible vaccine carrier for the oral vaccination of European badgers (*Meles meles*) against tuberculosis. *Vaccine* **2017**, *35*, 987-992, doi:10.1016/j.vaccine.2016.12.004.
6. Coutant, T.; Vergneau-Grosset, C.; Langlois, I. Overview of Drug Delivery Methods in Exotics, Including Their Anatomic and Physiologic Considerations. *Vet Clin North Am Exot Anim Pract* **2018**, *21*, 215-259, doi:10.1016/j.cvex.2018.01.006.
7. Boyd, B.J. Past and future evolution in colloidal drug delivery systems. *Expert Opin Drug Deliv* **2008**, *5*, 69-85, doi:10.1517/17425247.5.1.69.
8. Li, P.; Nielsen, H.M.; Mullertz, A. Oral delivery of peptides and proteins using lipid-based drug delivery systems. *Expert Opin Drug Deliv* **2012**, *9*, 1289-1304, doi:10.1517/17425247.2012.717068.
9. Aburahma, M.H. Bile salts-containing vesicles: promising pharmaceutical carriers for oral delivery of poorly water-soluble drugs and peptide/protein-based therapeutics or vaccines. *Drug Deliv* **2016**, *23*, 1847-1867, doi:10.3109/10717544.2014.976892.
10. Monteiro-Maia, R.; Ortigao-de-Sampaio, M.B.; Pinho, R.T.; Castello-Branco, L.R. Modulation of humoral immune response to oral BCG vaccination by *Mycobacterium bovis* BCG Moreau Rio de Janeiro (RDJ) in healthy adults. *J Immune Based Ther Vaccines* **2006**, *4*, 4, doi:10.1186/1476-8518-4-4.
11. Cosgrove, C.A.; Castello-Branco, L.R.; Hussell, T.; Sexton, A.; Giemza, R.; Phillips, R.; Williams, A.; Griffin, G.E.; Dougan, G.; Lewis, D.J. Boosting of cellular immunity against *Mycobacterium tuberculosis* and modulation of skin cytokine responses in healthy human volunteers by *Mycobacterium bovis* BCG substrain Moreau Rio de Janeiro oral vaccine. *Infect. Immun.* **2006**, *74*, 2449-2452, doi:10.1128/IAI.74.4.2449-2452.2006.
12. Skinner, M.A.; Keen, D.L.; Parlane, N.A.; Hamel, K.L.; Yates, G.F.; Buddle, B.M. Improving protective efficacy of BCG vaccination for wildlife against bovine tuberculosis. *Res. Vet. Sci.* **2005**, *78*, 231-236, doi:10.1016/j.rvsc.2004.07.007.
13. Buddle, B.M.; Aldwell, F.E.; Keen, D.L.; Parlane, N.A.; Yates, G.; de Lisle, G.W. Intraduodenal vaccination of brushtail possums with bacille Calmette-Guerin enhances immune responses and protection against *Mycobacterium bovis* infection. *Int. J. Tuberc. Lung Dis.* **1997**, *1*, 377-383.
14. Gormley, E.; Ni Bhuachalla, D.; O'Keeffe, J.; Murphy, D.; Aldwell, F.E.; Fitzsimons, T.; Stanley, P.; Tratalos, J.A.; McGrath, G.; Fogarty, N., et al. Oral Vaccination of Free-Living Badgers (*Meles meles*) with Bacille Calmette Guerin (BCG) Vaccine Confers Protection against Tuberculosis. *PloS one* **2017**, *12*, e0168851, doi:10.1371/journal.pone.0168851.
15. Nol, P.; Lyashchenko, K.P.; Greenwald, R.; Esfandiari, J.; Waters, W.R.; Palmer, M.V.; Nonnecke, B.J.; Keefe, T.J.; Thacker, T.C.; Rhyan, J.C., et al. Humoral immune responses of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis* BCG vaccination and experimental challenge with *M. bovis*. *Clinical and vaccine immunology : CVI* **2009**, *16*, 323-329, doi:10.1128/CVI.00392-08.

16. Nugent, G.; Yockney, I.J.; Whitford, E.J.; Cross, M.L.; Aldwell, F.E.; Buddle, B.M. Field Trial of an Aerially-Distributed Tuberculosis Vaccine in a Low-Density Wildlife Population of Brushtail Possums (*Trichosurus vulpecula*). *PloS one* **2016**, *11*, e0167144, doi:10.1371/journal.pone.0167144.
17. Gou, J.; Liang, Y.; Miao, L.; Chao, Y.; Zhang, Y.; Yin, T.; He, H.; Tang, X. The promoting effect of enteric materials on the oral absorption of larotaxel-loaded polymer-lipid hybrid nanoparticles. *Eur. J. Pharm. Sci.* **2018**, *124*, 288-294, doi:10.1016/j.ejps.2018.09.002.
18. Oshi, M.A.; Naeem, M.; Bae, J.; Kim, J.; Lee, J.; Hasan, N.; Kim, W.; Im, E.; Jung, Y.; Yoo, J.W. Colon-targeted dexamethasone microcrystals with pH-sensitive chitosan/alginate/Eudragit S multilayers for the treatment of inflammatory bowel disease. *Carbohydr Polym* **2018**, *198*, 434-442, doi:10.1016/j.carbpol.2018.06.107.
19. Longet, S.; Aversa, V.; O'Donnell, D.; Tobias, J.; Rosa, M.; Holmgren, J.; Coulter, I.S.; Lavelle, E.C. Thermostability of the coating, antigen and immunostimulator in an adjuvanted oral capsule vaccine formulation. *Int. J. Pharm.* **2017**, *534*, 60-70, doi:10.1016/j.ijpharm.2017.10.013.
20. Srivastava, A.; Gowda, D.V.; Madhunapantula, S.V.; Siddaramaiah. Development and Efficacy Assessment of an Enteric Coated Porous Tablet Loaded With F4 Fimbriae for Oral Vaccination of Piglets against F4+ Escherichia coli Infections. *Curr Drug Deliv* **2016**, *13*, 121-130.
21. Stadler, M.; Viernstein, H. Optimization of a formulation containing viable lactic acid bacteria. *Int. J. Pharm.* **2003**, *256*, 117-122.
22. Huyghebaert, N.; Vermeire, A.; Neirynck, S.; Steidler, L.; Remaut, E.; Remon, J.P. Development of an enteric-coated formulation containing freeze-dried, viable recombinant *Lactococcus lactis* for the ileal mucosal delivery of human interleukin-10. *Eur. J. Pharm. Biopharm.* **2005**, *60*, 349-359, doi:10.1016/j.ejpb.2005.02.012.
23. Levine, M.M.; Ferreccio, C.; Abrego, P.; Martin, O.S.; Ortiz, E.; Cryz, S. Duration of efficacy of Ty21a, attenuated *Salmonella* typhi live oral vaccine. *Vaccine* **1999**, *17 Suppl 2*, S22-27.
24. de Barros, J.M.S.; Scherer, T.; Charalampopoulos, D.; Khutoryanskiy, V.V.; Edwards, A.D. A laminated polymer film formulation for enteric delivery of live vaccine and probiotic bacteria. *J. Pharm. Sci.* **2014**, *103*, 2022-2032, doi:10.1002/jps.23997.
25. Ratliff, T.L.; Ritchey, J.K.; Brandhorst, J.; Hanna, M.G., Jr. Time-dependent aggregation of reconstituted BCG vaccine. *J. Urol.* **1994**, *152*, 2147-2150.
26. Gheorghiu, M.; Lagranderie, M.; Balazuc, A.M. Stabilisation of BCG vaccines. *Dev. Biol. Stand.* **1996**, *87*, 251-261.
27. Gheorghiu, M.; Lagrange, P.H.; Fillastre, C. The stability and immunogenicity of a dispersed-grown freeze-dried Pasteur BCG vaccine. *J. Biol. Stand.* **1988**, *16*, 15-26.
28. Groves, M.J. BCG: the Past, Present and Future of a Tuberculosis Vaccine. *J. Pharm. Pharmacol.* **1997**, *49 (Suppl. 1)*, 7-15.
29. Rezwan, M.; Laneelle, M.A.; Sander, P.; Daffe, M. Breaking down the wall: fractionation of mycobacteria. *J. Microbiol. Methods* **2007**, *68*, 32-39, doi:10.1016/j.mimet.2006.05.016.
30. Jin, T.H.; Nguyen, L.; Qu, T.; Tsao, E. Improved formulation and lyophilization cycle for rBCG vaccine. *Vaccine* **2011**, *29*, 4848-4852, doi:10.1016/j.vaccine.2011.04.056.
31. Ohtake, S.; Wang, Y.J. Trehalose: current use and future applications. *J. Pharm. Sci.* **2011**, *100*, 2020-2053, doi:10.1002/jps.22458.