# Supplementary Materials: Toltrazuril-Loaded Polymeric Nanocapsules as a Promising Approach for the Preventive Control of Coccidiosis in Poultry

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**Table S1.** Primers forward (F), reverse (R) and probes (P) used for *Eimeria* identification.

| Species | Sequence source | Primer and probe sequences | Reference |
|---|---|---|---|
| *E. acervulina* | Ac-AD18-953 | F: GCAGTCCGATGAAAGGTATTTG<br>R: GAAGCGAAATGTTAGGCCATCT<br>P: [6FAM] ACAGTCCCCGCTGATGGTGTAACG[BHQ1] | 15 |
| *E. maxima* | EmMIC1 | F: TCGTTGCATTCGACAGATTC<br>R: TAGCGACTGCTCAAGGGTTT<br>P: [6FAM] ATTGTCCAGCCAAGGTTCCCTTCG[BHQ1] | 15 |
| *E. tenella* | Tn-E03-1161 | F: TCGTCTTTGGCTGGCTATTC<br>R: CAGAGAGTCGCCGTCACAGT<br>P: [6FAM] CTGGAAAGCGTCTCCTTCAATGCG[BHQ1] | 15 |
| *E. spp* | 5S rRNA | F: TCATCACCCAAAGGGATT<br>R: TTCATACTGCGTCTAATGCAC<br>P: [6FAM] CGCCGCTTAACTTCGGAGTTCAGATGGGAT [BHQ1] | 16 |
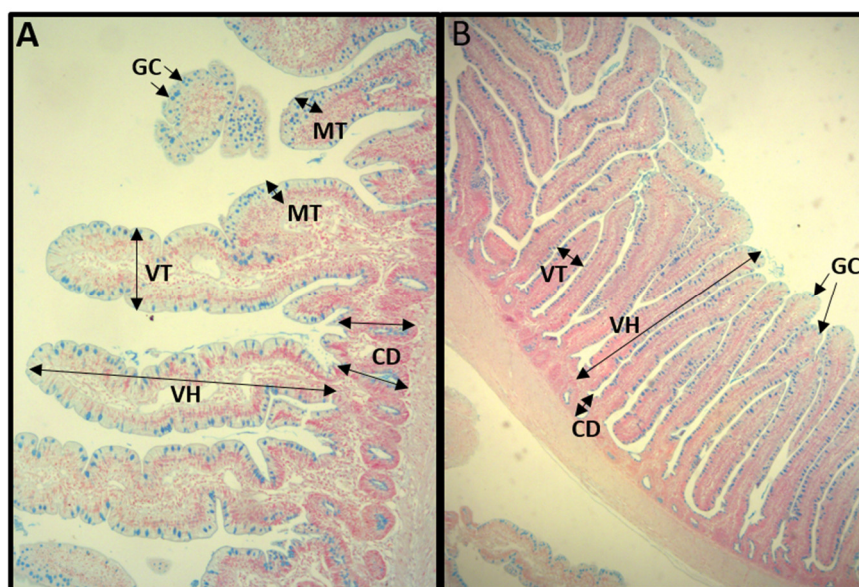


**Figure S1.** Small intestine stained with Alcian Blue & PAS. Cells stained in blue are goblet cells (GC). VH: villus height; CD: crypt depth; VT: villus thickness; MT: mucosal thickness. Magnification, 200× (**A**) and 100× (**B**).
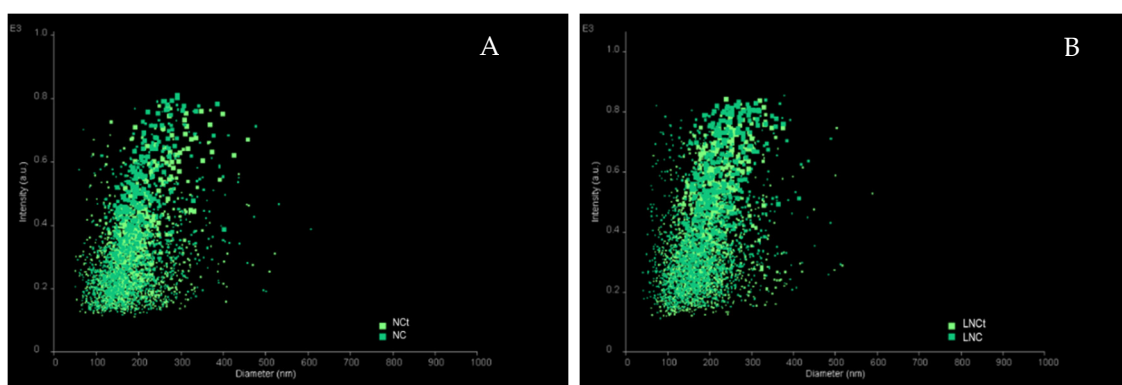
**Figure S2.** Light intensity graph obtained from particle tracking analysis. (**A**) Eudragit® S100 nanocapsules with toltrazuril (NCS100t) or not (NCS100); (**B**) Lipid core nanocapsules with toltrazuril (LNCt) or not (LNC).
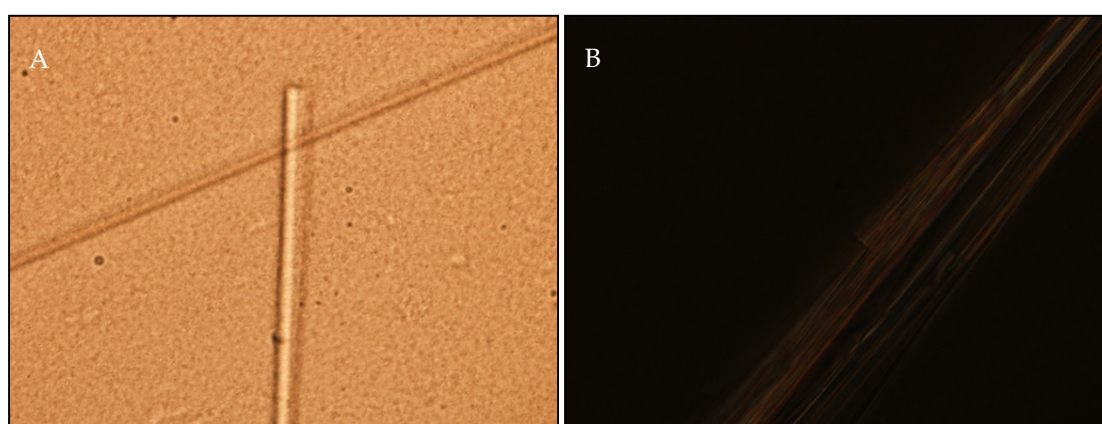


**Figure S3.** Photomicrographs obtained from the NCS100t formulation after 60 days of storage under refrigeration and keeping the formulation at rest. (**A**) normal light, (**B**) polarized light filter.

**Table S2.** Physicochemical parameters of nanoformulations containing toltrazuril after exposure to simulated gastric and intestinal media.

| Parameter | | LNC | NCS100 | LNCt | NCS100t |
|---|---|---|---|---|---|
| z-average (nm) | Before | 207 ± 1 | 154 ± 1 | 181 ± 2 | 151 ± 2 |
| | Gastric step | 211 ± 3 | 152 ± 1 | 178 ± 1 | 151 ± 2 |
| | Intestinal step | 207 ± 9 | 159 ± 1 | 176 ± 2 | 154 ± 3 |
| PDI | Before | 0.145 ± 0.012 | 0.133 ± 0.013 | 0.091 ± 0.010 | 0.116 ± 0.018 |
| | Gastric step | 0.171 ± 0.036 | 0.157 ± 0.021 | 0.100 ± 0.013 | 0.122 ± 0.006 |
| | Intestinal step | 0.145 ± 0.044 | 0.140 ± 0.063 | 0.095 ± 0.008 | 0.097 ± 0.009 |
| Content (%) | Before | n.d. | n.d. | 100* | 100* |
| | Gastric step | n.d. | n.d. | 105.24 ± 7.94 | 92.20 ± 2.23 |
| | Intestinal step | n.d. | n.d. | 99.61 ± 2.41 | 89.98 ±1.58 |

Values represent mean ± SD (n=3). LNC = blank lipid core nanocapsules; NCS100 = blank Eudragit® S100 nanocapsules; LNCt = toltrazuril-loaded lipid core nanocapsules; NCS100t = toltrazuril-loaded Eudragit® S100 nanocapsules formulations; n.d. = not determined. *toltrazuril concentration before exposure to gastric and intestinal media was considered 100%.

**Table S3.** Nanotechnological properties of formulations after dilution in water.

| | | LNCt | | LNC | |
|---|---|---|---|---|---|
| | | 0 h | 24 h | 0 h | 24 h |
| Particle diameter (nm) | 1 (1:10.5) | 180.2 ± 4.3 | 179.3 ± 1.9 | 210.7 ± 2.5 | 207.7 ± 2.1 |
| | 2 (1:21) | 180.1 ± 2.6 | 179.0 ± 0.6 | - | - |
| | 3 (1:42) | 177.9 ± 1.2 | 179.0 ± 1.8 | - | - |
| Drug content (%) | 1 (1:10.5) | 109.00 ± 5.18 | - | - | - |
| | 2 (1:21) | 106.31 ± 6.76 | - | - | - |

| | 3 (1:42) | 103.71 ± 10.50 | - | - | - |
|---|---|---|---|---|---|
| Association efficiency (%) | 1 (1:10.5) | 99.88 ± 0.07 | 99.93 ± 0.01 | - | - |
| | 2 (1:21) | 99.80 ± 0.08 | 99.86 ± 0.03 | - | - |
| | 3 (1:42) | 99.26 ± 0.91 | 99.74 ± 0.03 | - | - |

Values represent mean ± SD (n=3). LNC = blank lipid core nanocapsules; LNCt = toltrazuril-loaded lipid core nanocapsules.
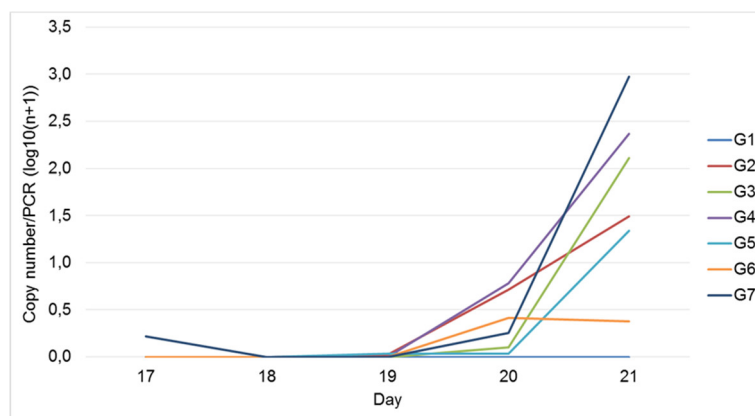


**Figure S4.** Copy number/PCR of *Eimeria spp.* in cage excreta through days 17 to 21 of the experiment. G1 = control (−); G2 = control (+); G3 = Baycox® (7 mg/kg/day); G4 = LNCt (7 mg/kg/day); G5 = LNCt (3.5 mg/kg/day); G6 = LNCt (1.75 mg/kg/day); G7 = LNC (volume equivalent to G4).
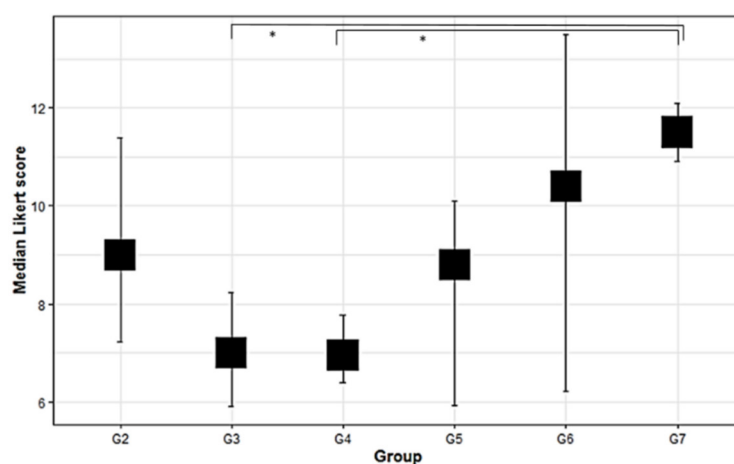


**Figure S5.** Plot of median Likert score versus group. Error bars indicate the 95% confidence intervals for the median with the percentile method. *Adjusted p-value <0.05. G2 = control (+); G3 = Baycox® (7 mg/kg/day); G4 = LNCt (7 mg/kg/day); G5 = LNCt (3.5 mg/kg/day); G6 = LNCt (1.75 mg/kg/day); G7 = LNC (volume equivalent to G4).