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1 **Highly Sensitive Detection of *Campylobacter spp.* in Chicken Meat using a Silica**
2 **Nanoparticle Enhanced Dot Blot DNA Biosensor**

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19 **Abstract**

20 Paper-based DNA biosensors are powerful tools in point-of-care diagnostics since they are
21 affordable, portable, user-friendly, rapid and robust. However, their sensitivity is not always as
22 high as required to enable DNA quantification. To improve the response of standard dot blots,
23 we have applied a new enhancement strategy that increases the sensitivity of assays based on
24 the use of biotinylated silica-nanoparticles (biotin-Si-NPs). After immobilization of a genomic
25 *Campylobacter* DNA onto a paper membrane, and addition of a biotinylated-DNA detection
26 probe, hybridization was evidenced using streptavidin-conjugated to horseradish peroxidase
27 (HRP) in the presence of luminol and H₂O₂. Replacement of the single biotin by the biotin-Si-
28 NPs boosted on average a 30 fold chemiluminescent read-out of the biosensor. Characterization
29 of biotin-Si-NPs onto a paper with immobilized DNA was done using a scanning electron
30 microscope. A limit of detection of 3 pg/μL of DNA, similar to the available qPCR kits, is
31 achieved, but it is cheaper, easier and avoids inhibition of DNA polymerase by molecules from
32 the food matrices. We demonstrated that the new dot blot coupled to biotin-Si-NPs successfully
33 detected *Campylobacter* from naturally contaminated chicken meat, without needing a PCR
34 step. Hence, such an enhanced dot blot paves the path to the development of a portable and
35 multiplex paper based platform for point-of-care screening of chicken carcasses for
36 *Campylobacter*.

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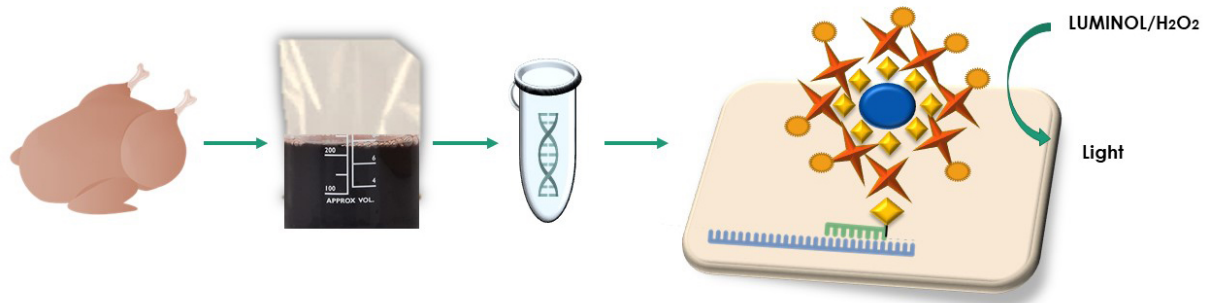
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43 **Graphical abstract**



44

45 **Keywords:** *Campylobacter*, DNA dot blot, Si-nanoparticles, Food safety, Multiplex
46 bacterial detection.

47

1. Introduction

48 *Campylobacter* is considered the most common bacterial cause of human gastroenteritis
49 in the world. The World Health Organization (WHO) estimates that 550 million people fall ill
50 every year from gastroenteritis, of which 220 million are children (WHO 2020). This zoonosis
51 is transmitted mostly through exposure to under-cooked poultry products (50-80% cases), and
52 in a minor way, to contaminated milk, vegetables and water (ECDC 2018; Hermans et al. 2012;
53 Humphrey et al. 2007). Campylobacteriosis in humans is usually a self-limiting condition
54 involving bloody diarrhea, abdominal cramping, nausea and fever, which can last up to two
55 weeks. In 1 % of cases, campylobacteriosis evolves to the Guillain-Barré syndrome, a severe
56 autoimmune disease that leads to death in 2–12 % of patients, depending on their age (Scallan
57 Walter et al. 2020). The overall economic burden of campylobacteriosis was estimated to about
58 EUR 3 billion/year in EU (ECDC 2018), and between US\$ 1.2 and 4 billion/year for the US
59 (Batz et al. 2014). To prevent the entry of *Campylobacter* contaminated broilers into the market,
60 the European Commission adopted a process hygiene criterion (EU 2017/1495) with the critical
61 limit of 1000 cfu/g of broiler meat or skin and obligatory systematic screening of broiler
62 carcasses for *Campylobacter* spp. (EU No 2017/625).

63 Identification and quantification of *Campylobacter* spp. rely on official, culture-based
64 methods, and bacterial biochemical/phenotypical characterization (Vizzini et al. 2019).
65 *Campylobacter* is highly infectious, with reported infective doses as low as 500 cells (Black et
66 al. 1988). To ensure the detection of one cell of *Campylobacter* in 25 g of food, an enrichment
67 step in Bolton broth for 24–48 h is needed, before isolation of the colonies by culturing (it takes
68 about 48–72 h) onto selective agar plates incubated in chambers for microaerophilic conditions.
69 *Campylobacter* is usually present in low numbers in food samples when compared to bacteria
70 like coliforms and Enterobacteriaceae making its isolation on common agar media difficult.
71 The official ISO 10272-1:2006 method for *Campylobacter* detection may provide false negative

72 results because of the possibility *Campylobacter* death during handling. Furthermore,
73 *Campylobacter* can enter in a viable but not cultivable (VBNC) status in food matrices, making
74 its detection based on culturing impossible (Vidic et al. 2017; Vizzini et al. 2019). Identification
75 of *Campylobacter* by the optical microscope is not easy as bacteria can change their spiral
76 distinctive shape into spherical or coccoid. Moreover, the current problem of official methods
77 is the use of a not enough selective medium, and emergence of bacterial resistance against the
78 antibiotic added to the enrichment broth.

79 Culture-independent molecular methods, such as PCR, and real-time PCR are used as
80 alternatives to colony growth (De Boer et al. 2015; Fontanot et al. 2014; Gosselin-Théberge et
81 al. 2016; Liu et al. 2017; Ricke et al. 2019). However, PCR-based methods may also provide
82 false-negative responses because of the sensitivity of DNA polymerase to inhibitors present in
83 food matrices and enrichment broths (Schrader et al. 2012; Vidic et al. 2019). Next generation
84 sequencing (NGS) which enables sequencing of the entire bacterial genome in a relatively short
85 time has started to be used as a tool for the identification of infectious bacteria. However, NGS
86 can hardly be routinely used at farms and slaughterhouse because it requires special equipment
87 and highly trained personnel for data interpretation (Gosiewski et al. 2017).

88 Biosensors for detection of *Campylobacter* show marked advantages over traditional
89 methods in terms of rapidity, facility utilization and cost-effectiveness (Manzano et al. 2015;
90 Masdor et al. 2016; Morant-Miñana and Elizalde 2015; Vidic et al. 2017; Vidic et al. 2019;
91 Vizzini et al. 2019; Yang et al. 2013). However, today no commercial biosensor is available to
92 detect *Campylobacter* in food matrices, mainly due to the difficulty of attaining a high
93 sensitivity.

94 Due to significant improvement in biosensor technology over the last two decades,
95 applications of paper-based sensors in pathogen detection are increasing. Such devices seem to
96 meet ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust,

97 Equipment free and Deliverable) recommended by the WHO for point-of-care diagnostics.
98 Paper as a substrate has the advantage of being inexpensive, lightweight, and easily enables
99 multiplex analysis (Dincer et al. 2019). Various paper-based methods to detect foodborne
100 pathogens have been reported including enzymatic-, immuno- and DNA/RNA- tests (Jokerst et
101 al. 2012; Morales-Narváez et al. 2015; Russell et al. 2017). Tests that target nucleic acids are
102 among the most accurate and specific although they are usually associated with a PCR step,
103 including isothermal amplification, to allow for detection of pathogens in low titers (Trinh et
104 al. 2020; Vidic et al. 2019).

105 Here, we coupled functionalized silica nanoparticles (Si-NPs) to a paper based DNA dot
106 blot test to enable sensitive *Campylobacter* nucleic acid detection without a pre-amplification
107 step. A highly specific DNA probe that recognizes the 16S rRNA gene of the most prevalent
108 *Campylobacter* spp. causing infections (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) was used
109 in the test. Si-NPs decorated with biotin (biotin-Si-NPs) were employed to enhance the
110 streptavidin–HRP chemiluminescent signal read-out. Our study illustrates the proof-of-
111 concept that the collective effect of biotin-Si-NPs could become an effective means for
112 increasing the sensitivity of the cost-effective, specific and easy-to-perform dot blot assay.

113 **2. Material and Methods**

114 *2.1 Materials and reagents*

115 Streptavine-HRP, Proclin, acetonitrile, 1,8-Diazabicyclo[5.4.0]undéc-7-ène (DBU),
116 Controlled pore glass (120-200 Mesh, CPG-3000 Å) were purchased from Sigma-Aldrich
117 (Saint Quentin Fallavier, France). Fluorescent rhodamine B silica NPs (Si-NPs, diameter~50
118 nm) were supplied by Nano-H (Saint Quentin Fallavier, France). DNA phosphoramidite
119 synthons and all DNA-synthesis reagents were purchased from Glen Research (Sterling,
120 Virginia, USA). Phosphate buffered saline (PBS) was purchased from Dominique Dutscher

121 (Brumath, France). Amersham Hybond-N+ nylon and Amercham Hybon-XL nitrocellulose
122 membranes were purchased from ThermoFisher (Illkirch, France).

123 All bacterial media and supplements used were from Oxoid (Milan, Italy), except for
124 the Violet red Bile glucose (VRBG) agar and Coli ID medium that were purchased from
125 Biomerieux (Bagno a Ripoli, Italy). Triton, SDS, NaCl, trizma-base, phenol, chloroform,
126 bacteriological peptone and isoamyl alcohol were purchased from Sigma (Milan, Italy). A 36-
127 mer oligonucleotide related to the 16S gene encoding for ribosomal *Campylobacter* RNA of *C.*
128 *jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (base location: 72-108) was used as a recognition
129 element (probe named CampyP3). It is interesting to note that the probe matches in three points
130 for each *Campylobacter* genome at 100%. CampyP3 was labeled at 5' with biotin for
131 chemiluminescent dot blot assay development. The probe was tested *in silico* by the
132 OligoAnalyzer3.1 (<https://eu.idtdna.com/calc/analyzer>), the Ampliflix software, Fast PCR 6.1
133 and Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). An ssDNA sequence complementary to the
134 CampyP3 probe, of the same length as the probe was named CP3 and used as positive control.
135 Two ssDNA sequences of the same length as the CP3, but not complementary to CampyP3,
136 were named PR and PE, and used as negative controls to study selectivity of the sensor through
137 their hybridization with the probe. PR was designed by mismatching positions of nucleic acids
138 of the CP3 sequence, while PE corresponded to the sequence of *E. coli* (accession number
139 527445.1) (base location: 338-376 for *E. coli*), which shows some similarities to
140 *Campylobacter*. These sequences, reported in Table 1S, were provided by Sigma-Aldrich (Saint
141 Quentin Fallavier, France) as lyophilized powers. All solutions were prepared using Milli-Q
142 water.

143 2.2 Bacterial strains

144 Bacteria used in this study are listed in Table 2S. *Campylobacter* strains were grown under
145 microaerophilic conditions (5% O₂, 10 % CO₂ and 80% N₂, generated with a Sachet Oxoid™

146 CampyGen™ 2.5 L (Oxoid, Italy) in anaerobic gas jars at 37°C for 48h, on Columbia blood
147 agar plates. *Campylobacter* isolates, both reference strains and strain isolates from chicken
148 samples, were subjected to Gram staining and optical microscope observations for cell
149 morphology and motility (Brucella broth, Thermofisher, Milan, Italy) after oxidase and catalase
150 tests.

151 All negative control strains were grown on their specific culture media at optimal conditions
152 and subjected to the same tests carried out for *Campylobacter* before DNA extractions.

153 2.3 Sample collection, plate count enumeration and selective isolation

154 Five chickens were purchased from local supermarkets in Italy. 10 g of chicken skin was
155 transferred into a filter-sterile stomacher bag with 40 mL saline-peptone water (8 g/L NaCl, 1
156 g/L bacteriological peptone) and homogenized in a Stomacher (PBI, Milan, Italy) for 90 s.
157 Aliquots of 0.1 mL were spread for the mesophilic aerobic count on Triptone Soya Agar, and
158 incubated at 30°C for 48 h, while 0.1 mL were spread on Agar Malt tetracycline at 30°C for 48
159 h to count yeast/molds. Aliquots of 1 mL were used with the pour plate method for enumeration
160 of Enterobacteriaceae in the VRBG agar (37°C for 24 h), and coliforms and *E. coli* in Coli ID
161 medium (37°C for 24 h). *Campylobacter* detection was performed according to the
162 conventional method ISO 10272-1:2006. For selective bacterial isolation, 10 µL of the Bolton
163 broth were streaked onto Skirrow agar plates, and mCCDA (modified charcoal-cefoperazone-
164 deoxycholade) plates and incubated at 41.5 °C for 48h, under microaerophilic conditions. One
165 colony was selected from mCCDA and streaked on two plates of Columbia blood agar. One
166 plate was incubated at 41.5 °C for 48 h in aerobic condition and one plate at 25 °C for 48 h in
167 microphilic condition. Growth on Skirrow served as a confirmation to proceed with the
168 identification steps.

169 2.4 DNA extraction from pure cultures and enrichment broths

170 DNA was extracted from reference strains and isolates from chicken samples according to
171 (Manzano et al. 2015). 2 mL of Bolton enrichment broth was centrifuged at 13,000 g at 4 °C
172 for 10 min, and bacterial pellets were washed three times with PBS 1X, and subjected to
173 extraction (Manzano et al. 2015). Extracted DNAs were rehydrated using 50 µL of sterile
174 distilled water and treated with RNase enzyme at 37°C for 1 h. Finally, DNA was quantified
175 using a Nanodrop™ 2000C (ThermoFisher Scientific, Milan Italy) spectrophotometer. The
176 concentration of DNA was adjusted to 100 ng/µL using sterile distilled water.

177 *2.5 Si-NP synthesis, labeling and quantification*

178 Si-NPs was functionalized by an innovative solid-phase synthesis technology which
179 enables functionalization of nanosized particles with DNA fragments, as reported previously
180 (Bonnet et al. 2018; De Crozals et al. 2012). Briefly, nanoparticle immobilization onto
181 controlled pore glass (CPG) allows a very high functionalization with a modified
182 oligonucleotidic based linker. The linker (sequence: dT10-PEG2-dT10) was synthesized using
183 an applied Biosystems 394 RNA/DNA synthesizer (Applied Biosystems). After grafting
184 nanoparticles on a CPG support, they were functionalized by automated synthesis using the
185 phosphoramidite chemistry. First, the linker, that allows a better accessibility of the functions
186 of interest, was synthesized. Second, the biotin group was incorporated. We controlled the
187 biotin incorporation to reach a 10% coupling yield. To do it, diluted solutions of biotin
188 phosphoramidite (10 mM) and tetrazole in acetonitrile (45 mM) were used and the coupling
189 time was reduced to 10 s. The biotin incorporation was monitored at 498 nm using
190 dimethoxytrityl quantification by an UV-visible spectrophotometer. Third, biotin-Si-NPs were
191 released from CPG by incubating the support in 1 mL of 0.1% (m/v) DBU in water-acetonitrile
192 1/1 (v/v). The DBU solution was stirred in a thermomixer at 22°C during 1 h before recovering.
193 A fresh DBU solution was added to the CPG suspension every hour. The release kinetics was
194 followed by quantifying the NP concentration in each DBU solution with an UV-visible

195 measurement at 560 nm. Released nanoparticles were washed with milli-Q water (1x 4 mL
196 then 3 x 2 mL) and concentrated on 30 K Amicon Ultra filter (5000 g, 10 min). Finally, the
197 amount of dT10-PEG2-dT10-10% Biotin strands per NP was estimated using a Varian Cary
198 100 Bio UV-visible spectrophotometer (Agilent Technologies) and a quartz cuvette of 1 cm
199 path length.

200 The amount of strands per NP was estimated using a Varian Cary 100 Bio UV-visible
201 spectrophotometer (Agilent Technologies) and a quartz cuvette of 1 cm path length. The amount
202 of linker grafted to nanoparticles was quantified by measuring absorbance in water (200 μ L) at
203 260 nm and 560 nm with a microplate reader (Perkin Elmer). The nanoparticle concentration
204 was estimated as described previously (Bonnet et al. 2018). To fit with this estimation, we
205 approximated a molar extinction coefficient to $163200 \text{ M}^{-1} \text{ cm}^{-1}$ that considers the epsilons of
206 the different parts of the sequence corrected by their corresponding coupling yield.

207 Functionalized NPs were observed under a transmission electron microscopy (TEM)
208 using a Philips CM120 instrument operating at an accelerate voltage of 120 kV (Centre
209 Technologique des microstructures, Lyon). Si-NPs were observed after deposition of 5 μ L of
210 diluted solution on a formvar-carbon coated copper grid followed by evaporation until dry.

211 *2.6 Scanning electron microscopy*

212 Samples were mounted on aluminum stubs (50 mm diameter) with carbon adhesive
213 discs (Agar Scientific, Oxford Instruments SAS, Gomez-la-Ville, France) and visualized by
214 field emission gun scanning electron microscopy (SEM FEG) as secondary and backscattered
215 electrons images (5 kV) under high vacuum conditions with a Hitachi SU5000 instrument
216 (Milexia, Verrières-le-Buisson, France). Sample preparation and scanning Electron
217 Microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2
218 (INRAE, Jouy-en-Josas, France).

219 *2.7 Sample immobilization and detection procedure*

220 Prior to immobilization onto the nylon membrane, extracted DNAs were denatured at 95
221 °C for 10 min, put immediately on ice and 1 µL spotted on the positively charged nylon
222 membrane (Amersham HybonTM-XL, GE Healthcare, France), which was exposed to UV at
223 254 nm for 10 min to fix DNA. The membrane was then soaked in a pre-wormed hybridization
224 buffer (0.5 M Na₂HPO₄, 0.5 M NaH₂PO₄, 10 mM EDTA, 1 % SDS, pH 7.5) at 65°C for 30 min
225 under gentle shaking. 4 ng/µL of the denatured biotin labeled CampyP3 probe (100 ng/µL) was
226 added to the hybridization buffer and left overnight at 65°C under gentle shaking to allow for
227 hybridization.

228 Subsequently, the membrane was washed twice with 0.1 % SDS, 300 mM SSC (Saline
229 sodium citrate, Meraudex, France) for 5 min and with 75 mM SSC for 15 min. The membrane
230 was transferred in the blocking buffer (0.1 % Tween, 0.1 % BSA, 0.03% Proclin, PBS, pH 7.4)
231 at room temperature for 15 min to saturate the surface. The membrane was finally incubated
232 with the blocking solution containing 0.7 µM streptavidin-HRP for 15 min at room temperature.
233 After washing with 0.1 % SDS, 150 mM SSC at room temperature for 5 min, the membrane
234 was incubated with 10⁶ biotin-Si-NPs/mL, PBS, pH 7.4 under gentle shaking at room
235 temperature, for 30 min. A 0.7 µM streptavidin-HRP in blocking solution was added after
236 washing and incubated for 30 min with shaking. The signal was then revealed using the
237 enhanced chemiluminescent substrate for detection HPR (Thermo Scientific, France). The
238 membrane was removed from the solution and observed under a ChemiDoc MP imaging system
239 (Biorad, France). Detection signals were quantified using Image LabTM software (Biorad). The
240 normalized value of a spot intensity was calculated by $(PI_0 - PI_n)/PI_0$, where PI_0 and PI_n
241 represent the pixel intensity obtained for detection of 0.1 ng/µL CP3 probe (standard) and the
242 experimental sample, respectively.

243 **Results and discussion**

244 *3.1. Preparation and characterization of biotin-Si-NPs*

245 Biotin-Si-NPs were prepared by an innovative solid-phase synthesis technology that
246 allowed for a very high functionalization. A hydroxyl group was attached to the Si-NP surface
247 by silanization to support the oligonucleotide linker synthesis on the NP surfaces, as previously
248 reported (Bonnet et al. 2018; Farre et al. 2010). In the final step, biotin groups were grafted to
249 the linker. TEM observations showed that the NP morphology was stable during synthesis (Fig.
250 1A). Functionalized biotin-Si-NPs had an average size of 50 ± 3 nm, as estimated from TEM
251 images (Fig. 1B). The absorbance measurements suggested that about 500 molecules of the
252 linker were attached to each NP, while dimethoxytrityl quantification indicated the biotin-
253 coupling yield of 10 %, which quantified biotin to about 50 per NP (Fig. 1C).

254 A quite narrow size distribution of functionalized NPs was confirmed by DLS
255 measurements. Si-NPs formed monodisperse aqueous solutions of particles with a
256 hydrodynamic diameter (R_H) of about 90 nm. Conjugation of biotin with the linker moieties
257 shifted R_H to about 120 nm (Fig. 1D). This increase is probably related to the hydration layer
258 around biotin units linked to arms bearing negatively charged groups. Biotin-Si-NP solutions
259 were stable at 4°C for at least two months (Supplementary Material, Figs. 1S and 2S).

260 *3.2. Concept of biotin-Si-NPs based DNA dot blot*

261 The principle of the DNA dot blot method for *Campylobacter* detection enhanced with
262 biotin-Si-NPs is described in Fig. 2. Initially, the target DNA was immobilized on a porous
263 nylon membrane by UV irradiation to enable crosslinking of DNA to the positively charged
264 surface. Thereafter, the DNA probe CampyP3 labeled with biotin was allowed to hybridize with
265 the target DNA. Hybridization was detected with a streptavidin-HRP conjugate in combination
266 with a chemiluminogenic substrate luminol in the presence of the activator H_2O_2 . In enhanced

267 detection, the streptavidin-biotin sandwich enabled attachment of biotin-Si-NPs to the DNA
268 probe, and consequently amplification of the detection signal compared to a single biotin.

269 3.3. Optimization of key parameters and analytical performances of the DNA dot blot sensor

270 The time and temperature of hybridization of the DNA probe with its target were
271 optimized as they can markedly influence the sensibility and selectivity of a DNA sensor. First,
272 hybridization of DNA was tested at room temperature, 44°C, 55°C and 65°C using
273 complementary and non-complementary short ssDNA targets. A complementary DNA target
274 sequence CP3 (positive control) was detected at all temperatures tested, but only at 65°C no
275 non-specific binding was obtained with negative controls, a truncated target sequence (PR) and
276 *E. coli* sequence (PE) (Fig. 3A). Second, hybridization was studied using 1 ng/μL CP3 at 65°C
277 for different times from 1 h to overnight. The overnight incubation provided the highest level
278 of hybridization and maximal signal intensity.

279 Other parameters that may affect hybridization, such as buffer compositions or the
280 paper support were also tested. The optimization criterion was the best signal-to-background
281 ratio. The nylon membrane allowed for better immobilization of DNA compared to
282 nitrocellulose. The effects of formamide (10, 25, 30, 35 and 50 %), DMSO (0 and 10%) and
283 SDS (0, 1 and 10 %) in the hybridization buffer were tested since these denaturing agents for
284 double stranded DNA may prevent nonspecific hybridization. The optimal hybridization buffer
285 contained SDS, and no DMSO or formamide. We determined that addition of 1 % SDS
286 enhanced the intensity of signals obtained and reduced the frequency of unspecific background
287 staining. The effect of SSC concentrations (0.5x, 1x and 2x) in the washing buffer was also
288 investigated. A 3-step washing procedure with two buffers (2xSSC, 0.1 % SDS and 0.5xSSC,
289 0.1 % SDS) lead to elimination of the background signal while maintaining high light intensity
290 of specific spots.

291 Detection of 1 ng/ μ L CP3 under the optimized conditions using various concentrations
292 of streptavidin-HRP and biotin-Si-NPs allowed selection of 25 ng/ μ L of streptavidin-HRP and
293 10^6 nanoparticles/mL for the chemiluminometric reaction. It is worth noting that the
294 chemiluminometric reaction itself does not significantly contribute to background staining as
295 the emission of light arises from the enzymatic reaction without any photonic excitation (Laios
296 et al. 2010).

297 3.4 Calibration curve

298 Calibration curves were obtained from quantification of chemiluminescent light
299 intensity for different CP3 concentrations, taken from at least three independent dots per
300 concentration. The linear range for the CP3 target was from 1 ng/ μ L to 0.1 ng/ μ L (with
301 correlation $R^2=0.98684$), and from 6 pg/ μ L to 0.1 ng/ μ L (with correlation $R^2=0.98935$), for
302 classical and biotin-Si-NP enhanced dot blot techniques, respectively (Fig. 3B-D). The limit of
303 detection (LOD) of 0.08 ng/ μ L and 0.003 ng/ μ L for classical and enhanced dot blot,
304 respectively, were calculated using $3 s/m$ formula, where 's' is the standard deviation of the
305 blank solution and 'm' is the slope of the linear calibration graph. Taking into account the
306 molecular weight of the CP3 of 14395.5, the calculated LOD was 5.5 nM and 0.2 nM, for
307 classical and enhanced dot blots, respectively. Consequently, biotin-Si-NPs allowed for almost
308 a 30 fold increase in chemiluminescent signal intensity compared to the classical dot blot. We
309 estimate LOD of about 600 cells considering that one *Campylobacter* cell contains 2 fg of
310 genomic DNA (Pacholewicz et al. 2013). The low LOD obtained suggests that this enhanced
311 dot blot method may be suitable for meat sample testing as the *Campylobacter* infection dose
312 is around 500 cells (2.5 pg DNA).

313 The proposed biosensor showed comparable or enhanced performance in relation to
314 recently reported DNA sensors for *Campylobacter* detection where the detection limit was 0.5

315 ng/ μ L in (Fontanot et al. 2014), 0.37 ng/ μ L in (Manzano et al. 2015) and 0.09 nM in (Morant-
316 Miñana and Elizalde 2015). The biotin-Si-NP enhanced dot blot reproducibility was estimated
317 to 5 % according to the signal obtained for detection of the same concentration of CP3.
318 Although our time of analysis is not the lowest reported for *Campylobacter* detection (Table 1),
319 our assay is, as far as we know, the first one for the *Campylobacter* detection that has a high
320 sensitivity and does not require the specific bacterial morphology or the background matrix.
321 The colorimetric aptasensor was reported to detect spiral *Campylobacter* cells but not spherical
322 or coccoid ones (Kim et al. 2018); whereas real-time PCR can be inhibited by molecules and
323 ions presented in enrichment broths or meat (Alves et al. 2016).

324 *SEM characterization of the biotin-Si-NPs dot blot*

325 SEM was applied to examine different detection steps in order to directly visualize
326 biotin-Si-NPs on the surface of the paper carrying hybridized DNA. The nylon surface before
327 DNA immobilization showed a typical membrane structure, including membrane pores (Figs.
328 4 and 3S). After *C. jejuni* DNA cross-linking to the nylon, the surface was occupied by a large
329 quantity of ssDNA. Compared to the surface of the bare membrane, the surface of membrane
330 carrying DNA became irregular showing increased morphological heterogeneity. ssDNA
331 molecules seemed to rest horizontally positioned over the surface (Fig. 4, right upper panel).
332 The membrane with hybridized DNA carrying biotin-Si-NPs (final detection step) had thicker
333 fibril structures than the ones with only immobilized ssDNA molecules (Fig. 4, lower panel).
334 Biotin-Si-NPs were easily detected on double strand DNA as small beads of about 50 nm in
335 diameter. It is worth to note, that because the DNA probe matches three points in the
336 *Campylobacter* genome, several biotin-Si-NPs may specifically bind to one DNA. Overall,
337 SEM images confirmed efficient DNA immobilization and hybridization by revealing that the
338 nylon surface became more complex after each stage of the detection process and that
339 hybridized DNA were decorated with nanoparticles of the expected size of 50 nm.

340 3.4. *Specificity and selectivity of detection*

341 To examine the selectivity and sensitivity of the biotin-Si-NPs dot blot, we tested
342 various strains of *Campylobacter* spp. for inclusivity, and 17 other bacterial species and 1 yeast
343 strain for exclusivity (Fig. 5). All strains were cultivated as monocultures and genomic DNA
344 were extracted as explained in the experimental part. Dot blot analyses were performed using
345 10 ng/ μ L of non-amplified extracted DNA. Before immobilization, all genomic DNA were
346 denatured for 5 min at 95°C to allow double strand opening. CP3 at 0.1 ng/ μ L was used as an
347 internal control to normalize signal intensities. The signal ratios for *C. jejuni*, *C. coli*, *C. lari*,
348 and *C. upsaliensis* over CP3 were higher than 1.0, with the membrane images exhibiting
349 obvious dots. Moreover, the intensity obtained for *C. jejuni* and *C. coli* were about 4 times
350 higher than that of the positive control. In contrast, the highest ratio among control DNA and
351 CP3 was only 0.6 for *S. enterica* (background staining). The obtained results highlight the
352 specificity of detection. In addition, the CampyP3 detection probe reacted with DNA of *C.*
353 *jejuni* and *C. coli* more efficiently than with DNA of other *Campylobacter* spp. tested. Our
354 results demonstrated that the biotin-Si-NPs enhanced dot blot platform is sensitive enough to
355 detect the whole DNA extracted from the most frequent *Campylobacter* spp. and could
356 distinguish between *Campylobacter* and other bacteria. This specificity of the CampyP3 probe
357 suggests that no nonspecific signal will be generated upon testing DNAs of competing total
358 mesophilic flora in chicken meat.

359 3.5. *Campylobacter* spp. detection in naturally contaminated chicken samples

360 To demonstrate the capabilities of our developed biosensor for possible real-world
361 applications, we have chosen to target the detection of *Campylobacter* in chicken meat samples.
362 The results of plate count enumeration for background bacteria in five chicken samples are
363 reported in Table 3S. The ISO 10272-1:2006 indicated that two out of five samples were

364 contaminated with *Campylobacter* (Table 4S). Indeed, only isolates from selective media
365 (mCCDA, Skirrow and Columbia agar) of samples C3 and C10 were confirmed for
366 *Campylobacter* by the temperature growth and motility test.

367 In dot blot analysis, the CP3 template (0.1 ng/ μ L) was used as a positive control. The
368 dot blot enhanced with biotin-Si-NP successfully detected two contaminated samples, C3 and
369 C10 (Fig. 6). The test achieved a relative specificity of 100 % due to the high specificity of the
370 CampyP3 detection probe. The proposed biotin-Si-NP enhanced dot blot method, thus,
371 specifically detected low levels of naturally present *Campylobacter* after enrichment and could
372 be considered for determination and detection of *Campylobacter* spp. in contaminated chicken
373 meat. Compared to the official ISO 11272: 2006 method, the new DNA dot blot biosensor is
374 less laborious, more cost-effective and time-saving. First, the highly selective DNA detection
375 probe enables selective detection of *Campylobacter* in the presence of meat background
376 bacteria, and thus no culturing on a selective agar is needed. Second, enhancement of the
377 detection signal intensity allows DNA detection without PCR pre-amplification.

378 **Conclusion**

379 This work describes the DNA dot blot method enhanced with biotin-Si-NPs for easy,
380 fast and reliable detection of *Campylobacter* spp. in contaminated chicken meats. The dot blot
381 test is widely used in molecular biology and genetics for detecting target DNA/RNA sequences.
382 This paper-based hybridization can easily analyze multiple samples inexpensively in a high-
383 throughput fashion but has limitation in a low sensitivity. We demonstrated that association of
384 highly functionalized biotin-Si-NPs with the chemiluminescent read-out of the dot blot
385 enhanced the signal about 30 times. Furthermore, biotin-Si-NPs are robust at working
386 temperature and stable in time. The LOD obtained was 3 pg/ μ L (0.2 nM). Such a low
387 concentration of detected DNA is close to values that can be detected by qPCR (Manzano et al.

388 2018; Vidic et al. 2019). Hybridization of immobilized genomic DNA with the CampyP3 probe
389 induced positive signals for different *Campylobacter* spp. responsible for human gastroenteritis,
390 while no background staining was observed with control samples. The developed system is a
391 promising tool for fast and cheap screening of poultry samples for the presence of
392 *Campylobacter* since detection is performed on bacterial DNA without a pre-amplification step.
393 Furthermore, our dot blot can be applied on DNA extracted by different extraction methods and
394 from various food matrices, as it is not sensitive to DNA polymerase inhibitors.

395 The proposed sensitive, miniaturized and multiplex paper-based test is simple to design
396 and could be used by the food industry and regulatory agencies for the detection of other
397 pathogens to monitor food quality. We believe that in the future it could be integrated into a
398 Lab-on-the chip based biosensor that comprises an automatized DNA extraction protocol and
399 a mobile phone camera (Kalligosfyri et al. 2019; Vidic et al. 2019).

400 Appendix A. Supplementary material

401 A list of oligonucleotides and bacterial strains used in this studies, stability of biotin-Si-
402 nanoparticles, as well as the results obtained by the plate count method on chicken samples are
403 available in the supplementary material associated with this article.

404

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416

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514

515

516 **Table 1.** Analytical parameters of methods for *Campylobacter spp.* detection.

Method	LOD	Analysis time	Reference
Culture-based methods	1 CFU / 25 g	≥ 5 days	(Vizzini et al. 2019)
Real-time PCR	3 x 10 ³ CFU/mL	0.5 to 4 h	(Alves et al. 2016)
Dot blot	0.37 ng/μL	24h	(Manzano et al. 2015)
Colorimetric aptasensor	7.2 × 10 ⁵ CFU/mL	30 min	(Kim et al. 2018)
Enhanced dot blot	3 pg/μL or 600 CFU	24h	This work

517

518 **Figure legends:**

519 **Figure 1.** (A) TEM images of Si-NPs before and after functionalization with linker and biotin.
520 (B) Histogram showing the average particle size 50 ± 3 nm before functionalization. (C)
521 Absorbance spectra of Si-NPs (before functionalization) and biotin-Si-NPs (after
522 functionalization). (D) DLS size plots of native and biotin functionalized Si-NPs in water.

523

524 **Figure 2.** Schematic representation of *Campylobacter* detection based on paper-based DNA
525 hybridization with a complementary biotinylated probe, and a streptavidin-HRP read-out
526 through dot blot (A). The signal was amplified using highly functionalized biotin-Si-NPs
527 instead of a single biotin (B).

528

529 **Figure 3.** (A) Dot blot detection of *Campylobacter* DNA sequence with biotin-Si-NP enhanced
530 read-out. Note that no signal was obtained with a truncated *Campylobacter* sequence (PR) nor
531 with an *E. coli* control sequence (PE). (B) Conventional dot blot detection of biotin labeled
532 CampyP3 probe using dilution of complementary *Campylobacter* sequence CP3 ranging from
533 $1\text{ ng}/\mu\text{L}$ to $78\text{ fg}/\mu\text{L}$. (C) Enhanced dot blot detection of CP3 ($0.1\text{ ng}/\mu\text{L}$ – $78\text{ fg}/\mu\text{L}$) using
534 CampyP3 probe. (D, E) Corresponding calibration curves were obtained by plotting the
535 chemiluminescent signal intensity of dots as a function of CP3 template concentrations.

536

537 **Figure 4.** SEM images of the basic nylon membrane, nylon membrane functionalized with a *C.*
538 *jejuni* DNA, and nylon membrane with CampyP3 hybridized with *C. jejuni* DNA and with
539 biotin-Si-NPs. Note that the size of visualized circular beads of about 50 nm (yellow shading)
540 corresponded to the estimated NP size in Fig 1B.

541

542 **Figure 5.** Inclusivity and exclusivity test results of the enhanced dot blot biosensor in pure
543 bacterial cultures. Signal intensity were normalized using an intern control (CP3 template).
544 Error bars represent the standard deviation of the mean from triplicates.

545

546 **Figure 6.** Schematic diagram of *Campylobacter* detection from chicken samples using the
547 enhanced dot blot and the official ISO 10272-1:2006 method. The ISO method involved steps
548 performed to obtain the results given in Table 4. Dot blot membrane shows representative dot
549 blot results obtained with naturally infected chicken samples (C3 and C10) and non-infected
550 chicken samples (C4, C5 and C6). CP3 template sequence (0.1 ng/μL) was used as a positive
551 control.

552