Electrochemical biosensors for the detection of pathogenic bacteria in food

Jordi Riu[1*], Barbara Giussani[2]

[1]Universitat Rovira i Virgili, Dept. Analytical and Organic Chemistry, C/ Marcel·lí Domingo 1, 43007 Tarragona. Spain

[2]Dipartimento di Scienza e Alta Tecnologia, Università degli Studi dell'Insubria, via Valleggio 9, 22100 Como, Italy

* To whom correspondence should be addressed. Universitat Rovira i Virgili, Department of Analytical Chemistry and Organic Chemistry, C/ Marcel·lí Domingo 1, 43007, Tarragona, Catalonia-Spain. E-mail: jordi.riu@urv.cat. Tel: +34 977558491; Fax: +34 977558446

Abstract

Biosensors for the detection of pathogenic bacteria in food are a promising alternative to conventional methods of analysis. The aim of this review is focussed on electrochemical biosensors reported in the very last years with application to food samples. The review highlights the performance parameters obtained by these sensors, with a critical discussion about their current and future trends, including future commercialization of these devices.

Keywords

Biosensor; Food analysis; Bacteria; Pathogen; Electrochemistry; Impedimetric; Amperometric; Voltamperometric

1. Introduction

Foodborne diseases are currently a serious concern that affect 600 million people worldwide [1]. Bacteria are the most common cause of these diseases. This problem does not concern only third-world countries since foodborne diseases are responsible for over 9.4 million illnesses every year in the USA [2], and a total of 5,079 foodborne outbreaks were reported in 2017 in the European Union [3]. Bioterrorism is also a concern because food can be an excellent carrier of pathogenic bacteria [4]. Due to the potential danger of foodborne pathogens and the fact that the infective dose of some of them is low, viable pathogenic cells of some species must be totally absent from food. This is the case for salmonella in accordance with the European Union Commission for Food Safety regulation (EC) 2073/2005 [5]. Considering all these hazards the demand for developing accurate, simple, rapid, low-cost and ideally portable analytical instruments that can make point-of-care analyses is constantly growing. Biosensors are analytical tools with the potential to meet these requirements.

The current review focuses on the most recent advances in electrochemical biosensors for the detection of pathogenic bacteria in food: papers published in the last 5 years are reviewed, since older papers have already been discussed in previous publications [6–12]. Only papers with food applications have been considered. After a brief introduction to some of the most important microorganisms involved in foodborne diseases (to understand their level of hazard and infective doses) and to conventional methods for detecting these microorganisms, we will focus

on electrochemical biosensors for detecting foodborne pathogens. We will finish by giving a critical point of view about the current state of biosensors and trying to envisage some trends for the future including current and future commercialization.

2. Pathogenic food bacteria

The most common foodborne diseases are those caused by *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni* and *Clostridium perfringens*, although the complete list of pathogenic food bacteria includes many other pathogens [13]. This review is not restricted to the above-mentioned microorganisms but also include biosensors relevant to other types of foodborne bacteria.

Escherichia coli (*E. coli*) is a Gram-negative rod-shaped coliform bacterium. Most *E. coli* are harmless but there are four pathogenic groups transmitted via contaminated food or water. Of these groups, enterohemorrhagic *E. coli* (EHEC) (including *E. coli* O157:H7, which accounts for around 75% of EHEC infections worldwide) is often involved in major foodborne outbreaks. The bacteria in the EHEC group produce the Shiga toxin that can cause such serious illnesses as bloody diarrhoea, blood-clotting problems, kidney failure, and death. Infections by EHEC can range from asymptomatic-to-mild diarrhoea to severe complications. The mortality rate of patients whose illness progresses to haemolytic uremic syndrome, which is characterized by acute renal failure, is between 3 and 5%. The infective dose for this group is very low (for instance, only 10 to 100 cells for *E. coli* O157:H7).

Salmonella is a Gram-negative rod-shaped bacterium. There are two species that can cause illness in humans: *Salmonella enterica*, the greatest public health concern, and *Salmonella bongori*. *Salmonella* can cause gastrointestinal illness (the infective dose can be as low as one cell depending on age and health) and typhoid fever (with an infective dose lower than 1000 cells). Typhoid fever may develop into septicaemia with the colonization of other tissues, which may be fatal.

Listeria monocytogenes is a Gram-positive rod-shaped bacterium. Although the number of people infected by *Listeria monocytogenes* is relatively small, this bacterium is one of the main causes of death from foodborne illnesses. The infective dose is undetermined because it depends on the strain, host and food matrix involved. One form of disease is a non-invasive gastrointestinal illness usually with no complications, but another form of disease is much more serious and can cause meningitis and septicaemia, with a case-fatality rate of 20-30% [14].

Campylobacter jejuni is a helical-shaped Gram-negative bacterium. Although other *Campylobacter* species also cause foodborne diseases, *Campylobacter jejuni* is responsible for more than 80% of *Campylobacter* infections and is estimated to be the third bacterial cause of foodborne diseases in USA. In general, the minimum infective dose is 10,000 cells, but in some cases it may be as low as 500 [15].

Clostridium perfringens is a Gram-positive spore-forming bacterium that produces the *Clostridium perfringens* enterotoxin. It is estimated to be the second bacterial cause of foodborne diseases in USA [16]. *Clostridium perfringens* causes two types of disease: one mild gastroenteritis and the much more severe enteritis necroticans, which is often fatal. The infective dose is above 1 million cells or 1 million spores/gram.

3. Biosensors for bacteria in food

The classical and standard method for identifying and quantifying bacteria is microbiological culture [17], which uses pre-enrichment and enrichment steps (with selective and non-selective media), isolation with appropriate culture media, biochemical confirmation and other steps. Most of the methods in the ISO 07.100.30 international classification of standards corresponding to ‘Food microbiology’ are culture-based and results can take between 3 and 7 days. Culturing techniques require considerable amounts of laboratory equipment, consumables, time and trained personnel.

Among the conventional alternatives to culturing techniques are PCR (polymerase chain reaction) and ELISA (enzyme-linked immunosorbent assay). PCR [18] is a nucleic acid amplification technique developed in the 1980s and widely used for identifying bacteria. The detection time in PCR-based methods [19] usually varies between 5 and 24 hours depending on specific PCR variation. Another limitation of PCR is that it has difficulty in differentiating between viable and non-viable cells. ELISA [20] is an immunological technique that uses an enzyme to detect the presence of an antibody or an antigen in a sample produced as a direct results of the bacterial presence. Both techniques often require enrichment or purification steps that can significantly lengthen the analysis time. PCR [19,21–23] and ELISA [24,25] have been extensively used for determining pathogenic bacteria in food samples. Several PCR and ELISA tests are available in the form of commercially prepared kits, the procedures are often highly automated, and the limits of detection can be as low as a few CFU/ml [11].

Biosensors are an alternative to conventional techniques for the detection of pathogenic bacteria in food. They are analytical devices (Figure 1) that consist of a biological part (receptor, sensing layer or recognition element) that can create a biorecognition event with the target analyte (pathogenic bacteria in our case), and this sensing layer is coupled to a suitable transducer to generate a measurable signal from the biorecognition event. In label-free biosensors, the transducer directly generates a measurable signal from the biorecognition event between the target analyte and the corresponding receptor. In label-based biosensors, the analyte is captured by the receptor but this biorecognition event does not generate a measurable signal. This type of biosensor requires a secondary molecule/aggregate (the label) to generate the measurable signal. Typical examples of label-based biosensors are sandwich assays in which a receptor (e.g. an antibody) is immobilized over the biosensor. This receptor captures the analyte of interest and then another receptor (e.g. a secondary antibody) attaches to the captured analyte. This secondary receptor is labelled with an appropriate molecule that generates the measurable signal. A typical label in electrochemical biosensors is the horseradish peroxidase enzyme (HRP) which catalyses the decomposition of the hydrogen peroxide added as a substrate to the medium. The main advantage of label-free biosensors is the simplicity of their construction and operation, while one of the advantages of label-based biosensors is the sensitivity offered by the labels [26].

In this review, we will focus on electrochemical biosensors for foodborne pathogens. A wide range of molecules – antibodies, aptamers, nucleic acids, etc. – can be used as the sensing layer, and this layer plays a key role in the selectivity and sensitivity of the device [27]. A wide range of materials can also be used in the transducer layer: among others, carbonaceous materials (graphene, carbon nanotubes, carbon ink, etc.), nanoparticles or nanowires.

Biosensors have a number of advantages over conventional analytical techniques: they require low volumes of sample, and have better selectivity, lower detection limits and faster response

129 time than conventional methods of analysis. They are generally compact, which makes them
130 prone to miniaturize [28] and they are easily integrated with standard electronic
131 microfabrication which makes it possible for devices to be wearable and be used for multiplex
132 sensing [29]. These characteristics are especially interesting in food analysis where rapid point-
133 of-care analysis [30] is often required. The introduction of new materials such as
134 nanostructured-materials [31] for surface modification or as transducer elements has also
135 offered a broad range of electrode modification possibilities which improve analytical
136 parameters such as sensitivity or limit of detection (LOD). An increasing number of reports are
137 being published on electrochemical biosensors [8,32].

138 We have included in this review those papers reporting a device (either a single device
139 containing all the electrodes or an electrochemical cell with separate electrodes that can be
140 integrated in a single device in a further development stage) in which the working electrode of
141 the biosensor contains in close contact both the transducer and receptor layer. We do not have
142 therefore considered analytical devices including methods of separation with the receptor not
143 attached to the biosensor (e.g. magnetic separation of the target bacteria using functionalized
144 nanomaterials) and further inoculation of the isolated bacteria over bare metallic electrodes for
145 electrochemical detection. We will begin with a rapid overview of the most common receptors
146 used and then we will move on to discuss the main types of electrochemical biosensors
147 according to the signal generated in the signal transduction.

148 **3.1 The receptor**

149 The receptor, most commonly an antibody, aptamer or DNA (although historically, enzymes
150 were the first receptors used in biosensors when Leland Clark used an amperometric enzyme
151 electrode to detect glucose in 1962 [33]) should have a high binding affinity with the target
152 analyte.

153 Antibodies (Ab), also called immunoglobulins (Ig), are large Y-shaped glycoproteins that can
154 recognize an antigen (molecules capable of stimulating an immune response) with a high
155 specificity [34]. Each antigen has different regions (epitopes) to which the antibody can bond.
156 Monoclonal antibodies are specific for a single epitope while polyclonal antibodies can bind
157 multiple epitopes in an antigen. Monoclonal antibodies are generated ex *vivo* using tissue-
158 culture techniques from identical cells that are clones from a single parent cell. Polyclonal
159 antibodies are produced by multiple cells of an animal in response to an injected infection.

160 Aptamers [35] are chains of oligonucleotides (single-strain DNA or RNA) or peptides that
161 specifically bind to a target molecule. DNA aptamers differ from typical DNA in the sense that
162 aptamers do not contain any genetic information. They simply adopt a 3D conformation in the
163 space that has a very high affinity for a specific target molecule. Aptamers are engineered
164 through repetitive cycles of in-vitro selection, well known as Systematic Evolution of Ligands by
165 Exponential Enrichment (SELEX) [36] to bind selectively to targets including small molecules,
166 proteins, virus or bacteria. Aptamers have a number of advantages over antibodies: lower costs,
167 shorter generation time, no batch-to-batch variability (especially compared to polyclonal
168 antibodies), better thermal stability and a wider range of conditions (including different pHs and
169 ionic strengths) in which they can operate. However, they can be degraded by nucleases. Single-
170 strain DNA sequences used as receptors in biosensors hybridize to specific gene sequences of
171 the pathogenic bacteria, requiring in this case the previous extraction of the bacterial DNA to be
172 detected [37].

173 The construction of the biosensor critically depends on the selection of the receptor but also on
174 the correct immobilization and orientation of the receptor on the transducer. The simplest
175 immobilization approaches include electrostatic or hydrophobic interactions with the
176 transducer [38]. Being rapid and simple, these approaches can have a significant impact on the
177 reproducibility of the construction and on the performance parameters. A more robust and
178 reproducible method of immobilization is the covalent attachment of the receptor to the
179 transducer. Carbodiimide and glutaraldehyde approaches are commonly used here [39].
180 Orientation [40] is also a critical step in the construction of the biosensor because it optimizes
181 the direct electron transfer that generates the signal and makes the recognition part of the
182 receptor available to the target analyte. The methods of immobilization previously discussed
183 usually mean that the receptor is randomly oriented, so immobilization methods with optimal
184 orientation of the receptor have been developed. One example of covalently-oriented
185 immobilization is cysteine-tagging by thiol-ene click chemistry [41] which forms stable thioether
186 bonds between the receptor and the transducer. Another strategy for oriented-immobilization
187 is the affinity binding approach, which takes advantage of the strong affinity between specific
188 couples of molecules (streptavidin-biotin, lectin-carbohydrates, metal cation-chelator, etc.): one
189 of the two needs to be attached to the transducer layer and the other to the receptor [39].

190

191 **4. Impedimetric biosensors**

192 Electrochemical impedance spectroscopy (EIS) is based on measuring the effective resistance of
193 an electrical circuit in response to the application of a sinusoidal alternating current (AC)
194 potential with a small excitation amplitude [42]. Impedance (Z), the frequency-dependant
195 resistance to current flow of a circuit element, can be expressed as the current-voltage ratio,
196 and Z is measured using EIS, in which the frequency of the applied sinusoidal potential varies.
197 The applied voltage amplitude is of the order of 10 mV or less. EIS is very sensitive at detecting
198 interfacial phenomena and therefore it can be used for the characterization of the different
199 steps in the construction of a biosensor involving modification of its surface, and for the
200 construction of the calibration curve where different amounts of the target analyte are bound
201 to the surface of the biosensor. To extract relevant parameters from EIS, the raw data are usually
202 fitted to a system modelling an equivalent circuit. The Randles model [43] is probably the most
203 common for EIS analysis. One of the most significant advantages of impedimetric biosensors is
204 their ability to perform label-free detection [44], although labelling can also be used in
205 impedimetric biosensors to increase selectivity or sensitivity.

206 The electrochemical cell is composed by a working electrode that directly or indirectly responds
207 to the analyte (pathogen) concentration, a counter or auxiliary electrode (often gold, platinum
208 or carbon electrode), and a reference electrode (usually a Ag-AgCl electrode or a pseudo-
209 reference Ag-AgCl in case or miniaturized screen-printed electrodes). Ideally, the three
210 electrodes should be integrated in a single device using for instance screen-printed or
211 lithographic techniques, but many reviewed papers report three separated electrodes that could
212 be integrated in a single device in a further development step.

213 The reviewed impedimetric papers are summarized in Table 1, including some performance
214 parameters and pre-treatments applied to the food samples.

215 **4.1 Antibodies**

216 Malvano et al. [45] tested different techniques of immobilization and orientation of monoclonal
217 anti-*E. coli* O157:H7 antibodies over commercial gold screen-printed electrodes (best limit of
218 detection $10^{0.47}$ CFU/ml). Results on real samples (meat and milk incubated for 90 minutes
219 before detection) were comparable with those obtained with a commercial ELISA kit (recoveries
220 between 91%-105%). Helali et al. [46] reported a biosensor for the detection of *E. coli* K12. The
221 authors directly immobilized the antibodies over the gold electrode by physisorption. A linear
222 range from 10^3-10^5 CFU/ml (apparently calculated with two regression points) was reported.
223 Frozen chicken was spiked with bacteria, and qualitative results detecting contamination are
224 presented.

225 A microfluidic-based impedance biosensor for the detection of three different serotypes of
226 *Salmonella* has been recently presented by Jasim et al. [47]. The device consists of three
227 microchannels, each one with 10 Au/Cr interdigitated electrodes and functionalized with
228 antibodies for a specific serotype. The authors claim that future versions of the device will be
229 disposable, showing a market-oriented approach with is not-common in the reviewed papers.
230 To meet this objective the authors will probably have to set-up a compact version of the required
231 instrumentation that includes syringe pumping, a function generator and an impedance analyser
232 (Figure 2). Despite the elegant device proposed, no calibration line is presented in the paper.
233 Turkey and chicken samples were spiked with *Salmonella* and the lowest measured
234 concentration was 7 CFU/ml (value obtained from a parallel study with cell culture), being able
235 to differentiate between live and dead bacteria with high level of precision.

236 Mutreja et al. [48] isolated and purified antibodies against an outer membrane antigen for the
237 construction of a biosensor for the detection of *Salmonella* Typhimurium (S. Typhimurium).
238 Carbodiimide crosslinker chemistry was used to attach the antibodies to the layer of graphene-
239 graphene oxide embedded on commercial screen-printed carbon electrodes containing the
240 reference, working and counter electrode. The linear range was defined between 10^2-10^6
241 CFU/ml. The limit of detection is 1.04×10^1 CFU/ml for lichi juice and 1.07×10^1 for orange juice.
242 The incubation time with the bacteria is 10 minutes. Recoveries ranging between 94% and 119%
243 are reported.

244 Farka et al. [49] also developed a biosensor for the detection of S. Typhimurium. In this case, the
245 antibodies against the lipopolysaccharides of S. Typhimurium were cross-linked to the gold
246 surface of a commercial screen-printed electrode using glutaraldehyde, obtaining a linear range
247 from 10^5-10^8 CFU/ml with a limit of detection of 7×10^4 CFU/ml (standard deviation of blank
248 measurements). 10 minutes sonication and heat slightly improve the detection limit but
249 increases the non-specific interactions. The total time of analysis was 20 minutes (no recovery
250 values in real samples are reported).

251 A MEMS microfluidic-based biosensor for the detection of S. Typhimurium was recently
252 developed by Liu et al. [50]. The device consists of two detection regions (with the possibility of
253 functionalizing each zone for a specific bacterium) fabricated using SU8 microchannels. Each
254 detection region consists of gold micro-interdigitated electrodes functionalized with antibodies
255 towards the target bacterium (two different antibodies for *Salmonella* in this case) using a
256 suitable cross-linker. The device is not intended to quantify the number of pathogens in the
257 sample but for positive or negative confirmation. The detection limit is 300 CFU/ml in less than
258 one hour (30 minutes incubation and 30 minutes washing). Ready-to-eat turkey was spiked with
259 *Salmonella* and positive contamination was confirmed. The devices are suitable to be re-used
260 after an appropriate cleaning process, although the paper, oriented towards the food industry
261 that prefers disposable devices to avoid cross-contamination, did not check the reusability.

262 Primiceri et al. [51] reported a compact biochip device for the detection of *Listeria* 263 *monocytogenes* and *Staphylococcus aureus*. The miniaturized device consists of an array of 264 interdigitated gold electrodes fabricated using optical lithography. Multianalyte detection with 265 different functionalization schemes is thus possible. The electrodes were functionalized with 266 antibodies by means of carbodiimide chemistry using also protein A for a correct orientation of 267 the antibodies. Wide calibration curves of up to nine orders of magnitude were obtained for 268 both bacteria (with some doubts regarding the linearity, especially for *Listeria monocytogenes*). 269 No selectivity studies with other bacteria were reported. Pork sausages were spiked with *Listeria* 270 *monocytogenes* and *Staphylococcus aureus*, but without a proper quantification of the results. 271 An updated version of the device with microfluidic structures and similar performance 272 parameters was also reported for the same research group [52].

273 **4.2 Aptamers**

274 A recent review on aptamers reports that there are 58 entries of aptamer selection against 275 pathogenic microbes (including foodborne bacteria) as of 2017 [53]. It is therefore not surprising 276 that several authors use the same aptamer sequence described in the literature for the 277 construction of their biosensors. This is the case for the aptamer reported by Joshi et al. [54] 278 against *Salmonella enterica* serovars. Using this aptamer, Jia et al. [55] built a biosensor 279 consisting of reduced graphene oxide (rGO) and carboxy-modified multi-walled carbon 280 nanotubes (MWCNTs) electrodeposited on the surface of a glassy carbon electrode (GCE). The 281 biosensor took advantage of the well-known electron transfer properties of carbonaceous 282 materials and of the high specific surface area of MWCNTs, which should result in improved 283 sensitivities. It is difficult to compare the sensitivities of the different biosensors, even if the 284 same receptor is used, since some calibration lines are reported in global response values and 285 some other ones in relative response values. The biosensor exhibited a linear range from 7.5×10^1 286 to 7.5×10^5 CFU/ml with a detection time of 60 minutes. A detection limit of 25 CFU/ml based on 287 the blank standard deviation was reported. Chicken samples were spiked with different amounts 288 of S. Typhimurium and recovery values between 101.9% and 106.7% were reported.

289 The group of A. Turner developed two different impedimetric biosensors for the detection of S. 290 Typhimurium using the same aptamer. One of them [56] was fabricated onto screen-printed 291 electrodes by electrochemically grafting a diazonium-supporting layer, followed by chemical 292 immobilisation of the aminated aptamer. Bacteria were detected after 30 minutes of incubation 293 time and 15 minutes of washing. The linear range is from 10^1-10^8 CFU/ml, with a LOD of 6 CFU/ml 294 based on the blank standard deviation. The reported recovery values show unusually high values 295 between 300% and 440%. The same aptamer was also covalently immobilized to poly[pyrrole-296 co-3-carboxyl-pyrrole] copolymer over gold disk electrodes in a different impedimetric 297 biosensor [57]. When applied to filtered and diluted apple juice, the performance parameters 298 were similar than for the previous reported biosensor [56], including similar high recovery 299 values.

300 Ranjbar et al. [58] used the same aptamer [54] to detect S. Typhimurium by synthesizing 301 nanoporous gold (NPG) over a glassy carbon electrode covered by a gold film. The receptor was 302 incorporated by adding the thiolated aptamer and incubating it overnight. The analytical 303 parameters are similar than other previous biosensors with the same aptamer, with a limit of 304 detection of only a 1 CFU/ml based on the blank standard deviation. The biosensor was also able 305 to differentiate between live and dead cells. The pathogen was spiked to egg samples 306 (centrifuged and diluted) and the recovery values were between 84.6% and 109.1%. The

307 biosensor can be regenerated in NaCl solution for 1 hour, but no information about reusability
308 was reported.

309 Finally, the same aptamer was also used to develop a biosensor using carboxylated MWCNTs
310 electrodeposited over an indium tin oxide glass electrode [59]. The amino-modified aptamer
311 was then immobilized over the electrode. Two serovars of *Samonella enterica*, S. Typhimurium
312 and S. Enteritidis, were used to construct two calibration lines with similar dynamic ranges
313 ($6.7x10^1$-$6.7x10^5$ CFU/ml and $5.5x10^1$-$5.5x10^6$ CFU/ml respectively). Looking at the two
314 regression lines some doubts arise about the reported linearities. Raw chicken meat spiked with
315 *Salmonella* (serovar not specified) was used to assess the detection in food samples. Sample
316 preparation seems not straightforward and includes enrichment and incubation overnight and
317 centrifugation. Recoveries were not calculated, only positive contamination was reported and
318 assessed by parallel PCR comparison.

319 Pathania et al. [60] also developed a biosensor for the detection of S. Typhimurium, but in this
320 case the aptamer was selected by the authors using a modified cell-based SELEX method. Gold
321 nanoparticles were electrochemically deposited over commercial screen-printed electrodes,
322 and then the surface was decorated with thiolated aptamers. The calibration line showed a
323 linear range between 10^1–10^5 CFU/ml with a limit of detection of 10 CFU/ml. S. Typhimurium
324 was spiked on homogenized egg samples, and recovery values between 120% and 450% were
325 found. No time of analysis was reported.

326 Hills et al. [61] developed an aptamer-based biosensor for the detection of *Listeria*
327 *monocytogenes*. A base of nanoplatinum was formed by electrodeposition over commercial
328 Pt/Ir electrodes, followed by drop-casting of reduced graphene oxide and the formation of a
329 second layer of nanoplatinum. Chitosan was then electrodeposited forming chitosan
330 nanobrushes. The authors take advantage of the biomimetic chitosan properties using the vast
331 nanobrush borders for vastly increasing the contact area with target bacteria. Aptamers were
332 then conjugated over the surface of the electrode. Antibodies were also tested as receptors in
333 this biosensor, but the best results were obtained using aptamers. The linear range was reported
334 between 10^0-10^7 CFU/ml, with LOD of 3 CFU/ml. Vegetable broth was also used to build the
335 calibration line. No accuracy (for instance using spiked samples) was calculated using real
336 samples. The total detection time was of 17 minutes including sample exposure and testing.

337 In a similar scheme than [58], a glassy carbon electrode was modified with gold nanoparticles
338 by electrodeposition and then a thiolated aptamer against outer-membrane proteins of *Shigella*
339 *dysenteriae* was self-assembled [62]. The linear range was reported between 10^1 to 10^8 CFU/ml,
340 with a LOD of 10 CFU/ml. The incubation time with the target bacteria was 30 minutes. Diluted
341 milk samples (1:10) were spiked with the target bacteria, with recovery values between 93.3%
342 and 133.0%. Selectivity was tested with five different bacteria, including another strain of
343 *Shigella*.

344 **4.3 DNA**

345 All the reviewed DNA biosensors (in impedimetric and other electrochemical-based biosensors)
346 reported the calibration curve using concentration of DNA instead of concentration of bacteria.
347 Doubts remain about how to translate the concentration of ssDNA to concentration of bacteria
348 in the samples.

349 Izadi et al. [63] developed a DNA-based based biosensor for *Bacillus cereus* detection in milk and
350 infant formula. ssDNA was immobilized with a thiol linker on gold nanoparticles that were

351 electrodeposited over a pencil graphite electrode. The DNA extraction procedure includes the
352 use of a commercial DNA extraction kit requiring several steps and 2-3 hours (although not
353 reported in the paper). The calibration curve (comprising two linear ranges) was constructed
354 using the concentration of ssDNA, but a limit of detection of 10 CFU/ml was reported. The
355 biosensor was applied to the detection of *Bacillus cereus* in milk and infant formula, with
356 recoveries ranging between 97%-101% but related to DNA concentration, so no information is
357 given about the quantification of bacteria. Regeneration of the biosensor was obtained
358 immersing it in NaOH for 2 minutes, and the biosensor was successfully regenerated for 7 times.

359 Finally, a DNA biosensor for the detection of *Clostridium perfringens* was developed by Qian et
360 al. [64] immobilizing the ssDNA probe on a CeO_2 nanorods/chitosan modified glassy carbon
361 electrode surface via metal coordination. The sensor detects a concentration of $7.06x10^{-15}$ mol/L
362 of oligonucleotides (milk was spiked with oligonucleotides), but no information is given about
363 the detected concentration of bacteria.

364 **4.4 Other receptors**

365 Wu et al. [65] used a molecularly imprinted polymer, MIP [66] (the authors refers at it as
366 bacteria-imprinted polypyrrole, BIP) directly polymerized over a GCE substrate for the detection
367 of *E. coli* O157:H7. A linear range from 10^3 to 10^8 CFU/ml was reported and the authors took the
368 lower limit of the linear range as the limit of quantification of their method (10^3 CFU/ml). The
369 time of analysis was 1 hour (incubation time with the target bacteria).

370 Bhardwaj et al. [67] used bacteriophages [68] as receptors in the detection of *Staphylococcus*
371 *arlettae*. Bacteriophages were immobilized over commercial graphene screen-printed
372 electrodes. The linear range was reported between $2\text{-}2x10^6$ CFU/ml with a limit of detection of
373 2 CFU/ml (taking the lower limit of the linear range), and with a very fast incubation time of 2
374 minutes. Complete studies of precision, regeneration and stability are also presented. The
375 sensor can be regenerated at least for 5 times and the long-term stability was checked over a
376 period of 3 months. Apple juice samples were spiked, and the results of the analysis were
377 compared with colony counting, obtaining good agreement values between the two methods.

378

379 **5. Amperometric and voltammetric biosensors**

380 Amperometric detection of pathogens is based on measuring the current generated by redox
381 reactions at the electrode surface under a given potential. The redox reactions involve directly
382 or indirectly the analyte, and a linear relationship between the analyte concentration and the
383 measured current can be observed [69]. Voltammetry is an analytical technique where the
384 current is measured under a potential sweep. In a voltammogram, the intensity of a peak is
385 directly proportional to the concentration of the analyte, while the position of the peak
386 maximum depends on the chemical species involved in the redox reactions. Voltammetry
387 includes various types depending on the potential control. Differential pulse voltammetry (DPV),
388 cyclic voltammetry (CV) and square wave voltammetry (SWV) are the most employed in the
389 detection of pathogenic bacteria in food. Cyclic voltammetry is also often used to characterize
390 the surface and the different steps of functionalization in all types of biosensors. As in
391 impedimetric biosensors, amperometric and voltammetric biosensors use a three-electrode
392 system.

393 The reviewed amperometric and voltammetric articles are summarized in Table 2.

5.1 Antibodies

A label-free biosensor was developed by Dhull et al. for the detection of *Escherichia coli* O157:17 in milk [70]. The antibody was covalently bound to the surface of nanostructured NiO thin film matrix RF-sputtered on a conducting indium tin oxide (ITO) coated glass substrate. The chemical stability of NiO allowed the surface modification for covalent binding of the antibody, enhancing the biosensing response and giving a larger shelf life of the electrode, with a low cost if compared with other stable metals such as gold. For the construction of the calibration line the authors use CV, thus the sensor cannot be considered as "amperometric" as reported in the manuscript. The linear range is from 10-10^7 cells/ml with a LOD of 1 cell/ml, with an incubation time of 1h and a response time of 5s. Diluted milk samples (dilution factor not reported) were spiked with bacteria, obtaining recovery values between 91% and 109%. No information concerning the stability in time of the sensor is given.

Good performances were also achieved when CdS quantum dots encapsulated in a metal organic framework (MOF) were used as signal-amplifying tags by Zhong et al. [71]. A sandwich-type immunosensor was fabricated immobilizing anti-*E. coli* O157:H7 antibody on a poly-(p-aminobenzoic acid)-modified GCE electrode. Then zeolitic imidazolate framework-8 (ZIF-8) was used to prepare the CdS@ZIF-8 multi-core-shell particles, which were subsequently coated with polyethyleneimine and further modified with the antibody. During the DPV analysis, a HCl diluted solution was dropped on the immunoelectrode, allowing the release of Cd(II) and its quantitative determination by stripping analysis. The biosensor shows a wide linear interval - 10 to 10^8 CFU/ml - with a LOD of 3 CFU/ml in about 2h analysis. The sensor proved to be stable in 15 days when used every 3 days for the detection of the bacteria.

Several authors functionalized a graphene surface with gold nanoparticles (AuNPs), typically in biosensors involving sandwich strategies. Anyway, the functionalization of the graphene surface with nanoparticles may result in problems related to poor nanoparticle dispersion and authors usually investigate conducting materials to avoid the problem of the dispersion. Poly-(3,4-ethylenedioxythiophene) (PEDOT) and sulfonated graphene (SG-PEDOT-AuNPs) composites were successfully synthetized by Guo et al. *via* a one-step reaction [72]. *E. coli* antibody was used as capture antibody and HRP as the label in a typical sandwich scheme (Figure 3). Zou et al. developed an enzyme-free label biosensor using polypirrole (PPy) to disperse gold nanoparticles and ferrocene as the labelling of the detection antibody [73], obtaining a device able to detect *E. coli* in whole milk samples with LOD of 10 CFU/ml and recoveries between 96-104% (spiked samples). Mo et al. developed a screen printed carbon electrode employing polyaniline (PANI) [74]. The PANI film and AuNPs formed a composite, with the AuNPs assembling and functioning as a scaffold for immobilizing antibodies on the electrode surface. The electrode can be simply regenerated by sonication.

Zhang et al. developed amplified strategies to estimate quality deterioration in dairy products on the basis of *E. coli* contamination [75–77]. The biosensors were able to detect the bacteria in spiked fresh milk, fresh yogurt and expired yogurt with a minimum sample pre-treatment obtaining recoveries between 90%-112%. The response of the biosensors [77] seemed not to be influenced by the matrix, demonstrating the appropriateness of the device for the estimation of *E. coli* in dairy products (LOD between 35-60 CFU/ml).

An interesting label-free biosensor for the determination of *Escherichia coli* was developed by Guo et al. integrating for the first time the rolling circle amplification (RCA) and the peroxidase-mimicking DNAzyme amplification techniques into an electrochemical assay for the

439 determination of pathogens in milk samples (recoveries between 95-102%) over a gold substrate
440 [78]. Among the different signal enhancement capabilities, RCA is one of the most used [79]. It
441 is a simple and efficient isothermal enzymatic process that uses nucleases to generate long
442 single stranded DNA (ssDNA) or RNA. The functional nucleic acid unit (aptamer, DNAzyme) can
443 be replicated hundreds of times in a short period, and a lower LOD can be achieved if those units
444 are combined with an enzymatic reaction. In less than two hours and a half a LOD of 8 CFU/ml
445 was achieved (18 CFU/ml when calculated in real milk samples), with a linear range from 9.4 to
446 9.4×10^5 CFU/ml. The electrode proved to be stable for 10 days (90% of the initial current
447 retained) when stored at 4°C.

448 The excellent ability to support biomolecules and the high surface area of gold nanoparticles
449 was also used by Xiang et al. [80] in the construction of a biocompatible film in conjunction with
450 chitosan over a glassy carbon electrode. The film was functionalized with *Salmonella* antibody
451 in order to detect *Salmonella* in milk samples (errors around 10% when compared with the plate
452 counting method). A similar strategy was presented by Fei et al. [81] modifying a screen-printed
453 carbon electrode with an ionic liquid and anti-*Salmonella pullorum* antibodies to capture the
454 pathogen from spiked eggs and chicken meat (sample pre-treatment not reported). In both
455 cases [80,81], the sandwich was then built with a secondary antibody labelled with HRP. In the
456 first case [80] the biosensor reached a very good LOD of 5 CFU/ml, with a linear range between
457 10 and 10^5 CFU/ml in 4 hours including fabrication and analysis time (DPV). When describing the
458 selectivity of the sensor, the authors claimed that the biosensor was able to selectively detect
459 only *Salmonella*. However, even though *E. coli* (used as control microorganism) showed a very
460 low current value, it seemed to be not negligible. In the case of the screen-printed electrode
461 [81], the LOD is higher – 3×10^3 CFU/ml, with a linear range between 10^4-10^9 CFU/ml. The sensor
462 showed a good stability of 30 days when stored (4°C) and used every 5 days: 93.8% of their initial
463 signal was retained. When employed in real samples, it was able to detect 100% of the true
464 positive samples but only 87.5% and 80% of the true negative samples in chicken and eggs
465 respectively.

466 A similar approach developed by Alexandre et al. [82] allowed to detect S. Typhimurium in
467 skimmed and whole milk samples reaching the best detection limit ever reported so far for this
468 bacteria in amperometric biosensors (10 CFU/ml, linear range not reported) with a detection
469 time of 125 min (chronoamperometry). Anyway, the analytical performance evidenced a
470 qualitative behaviour of the sensor being able to detect the presence or absence of the
471 microorganism. The authors also investigated the selectivity of the sensor, but other bacteria
472 showed a current that was higher than 50% of the current measured for *Salmonella*.

473 15 minutes (LOD of 5 CFU/ml) are needed to detect S. Typhimurium with a novel double-layer
474 chitosan-AuNPs immunosensor immobilized with *Salmonella* plasmid virulence C antibody and
475 using HRP and thionine as the amplification system [83]. The amperometric sensor proved to be
476 stable for 13 days (86.5% of the initial current after 13 days), when stored at 4°C and used every
477 three days. Anyway, only not contaminated milk samples were analysed both with the sensor
478 and the plate methods. No proofs of the biosensor suitability for real samples analysis are given
479 in the manuscript.

480 Very good limits of detection and fast detection times were obtained in biosensors for the
481 detection of *Staphylococcus aureus*. A very recent work by Wang et al. [84] reports a label-free
482 biosensor which replicates the microscopic porous structure of butterfly wings. Butterfly wings
483 were used as a biological template to prepare a hierarchical mesoporous silica substrate that
484 was functionalized to crosslink antibodies with glutaraldehyde immobilized on a GCE. DPV and

485 EIS were employed founding a narrow interval between 10 and $2x10^3$ CFU/ml, with a LOD of 11
486 CFU/ml and a detection time of only 20 minutes using DPV, and a LOD of 12 CFU/ml using EIS.
487 Anyway, when testing the selectivity against other bacteria, the registered current values for the
488 interferent bacteria overcome 50% of the registered current values for *Staphylococcus aureus*.
489 Good results were also obtained by Bhardwaj et el. using a low-cost paper-based biosensor [85]
490 to detect the bacteria in spiked milk in only 30 minutes. A LOD of 13 CFU/ml and a good linear
491 range between $10\text{-}10^7$ CFU/ml (recovery in the milk samples not reported) were obtained using
492 DPV. Single-walled carbon nanotubes (SWCNTs) were conjugated with antibodies *via* covalent
493 bonding and deposited on a carbon paste electrode (CPE) onto a printed paper sensor, which
494 showed stability for over a month. A possible drawback to this combination can be found again
495 in the selectivity, as non-specific bacteria resulted in more than 50% of current signal compared
496 to the specific response of *Staphylococcus aureus*.

497 Finally, Lu et al. employed novel MWCNTs fibers as the electrode material in a disposable
498 biosensor for *Lysteria monocytogenes* [86]. With this labelled design, it is possible to detect the
499 pathogen between $10^2\text{-}10^5$ CFU/ml, with a detection limit of $1.07x10^2$ CFU/ml. The sensor
500 showed a high selectivity using diluted milk samples. However, no information concerning
501 quantitative analysis or recoveries are given in the manuscript. CV was used for the quantitative
502 determination, while in the title the authors define the biosensor as “amperometric”.

503 **5.2 Aptamers**

504 A recent article [87] by Wang et al. reports the determination of *E. coli* in licorice extract using
505 an aptamer-based biosensor over a gold electrode. When applied to diluted licorice extracts (4%
506 solid content) the comparison between the results obtained by the electrochemical sensor and
507 the reference method (plate counting method) showed a statistically significant difference at
508 low concentration values.

509 S. Thyphimurium was detected by Dinshaw and Muniandy [88,89] in raw chicken samples using
510 reduced graphene oxide (rGO) as the substrate for the conductive platform of the bioelectrodes.
511 Samples were homogenized and diluted in both cases. In the first biosensor, rGO and chitosan
512 were electrodeposited on a GCE and then functionalized with the thiolated aptamer (see [54]
513 for the used aptamer). In the second approach, for the first time a label-free biosensor was
514 developed using a new conductive layer consisting of rGO-azophlorine (AP) nanocomposite as
515 the sensing platform. The AP nanocomposite showed excellent electrochemical activity due to
516 the enhanced electron transfer of AP. With an assay time of only 10 minutes, a LOD of 10 CFU/ml
517 is achieved, with a wide linear range between $10\text{-}10^8$ CFU/ml. The aptasensor proved to be
518 adequate in real samples analysis.

519 S. Typhimurium in pure milk was also detected with an aptasensor employing gold nanoparticles
520 and RCA signal amplification strategies by Ge et al. [90]. AuNPs were first electrochemically
521 deposited on a gold electrode and then functionalized with the DNA capture probe via covalent
522 bonds, and then combined with the aptamer. 120 min was chosen as the optimum RCA reaction
523 time, while 60 minutes were selected as the optimum reaction time of the aptamer with the
524 microorganisms. A linear range was found between $20\text{-}2x10^8$ CFU/ml (DPV), with a LOD of 16
525 CFU/ml (18 CFU/ml when calculated in milk samples). No information concerning errors and
526 recoveries in real samples are given in the article. Gold nanoparticles were also successfully
527 employed by Li et al. [91] in an electrochemical aptasensor based on target-induced strand
528 displacement and gold amplification for the quantification of *Salmonella* in milk.

529 Dai et al. [92] described in a very recent article the use of UiO-67/graphene (a Zr-based MOF
530 combined with graphene) as an electrode substrate in aptamer-based assays. Combining this
531 substrate with aptamer-AuNPs-HRP conjugates, the device was able to detect S. Typhimurium
532 with a low limit of detection of 5 CFU/ml and (DPV).

533 A different amplification strategy was adopted by Pei et al. in two articles describing biosensors
534 for the detection of S. Typhimurium [93,94]. The best results were achieved using Exo III-assisted
535 multiple-cycle amplification. The amplification reaction was activated specifically by the
536 pathogen-aptamer binding. The sensor shows a wide linear range (6 orders of magnitude) and
537 a detection limit of 8 CFU/ml. The sensor proved to be selective against other bacteria and when
538 used in real samples (spiked milks diluted with PBS) gave very similar results to the results
539 obtained with the counter plate (differences less than 7.2%).

540 Teng et al. successfully employed RCA also for the determination of *Vibrio parahaemolyticus* in
541 spiked fish sample [95] (sample pre-treatment not described). In this case, a gold electrode
542 functionalized with the antibody that captures the target pathogen was incubated with the
543 aptamer. The hetero-sandwich biosensor was then constructed using a AuNP-detection probe
544 (AuNP conjugated with ssDNA) hybridized with RCA, and adding methylene blue as the
545 electrochemical DNA probe. The signal covers a wide linear range of $2.2\text{-}2.2\text{x}10^8$ CFU/ml,
546 reaching a detection limit of 2 CFU/ml. While the authors define the biosensor as
547 "amperometric", DPV was used to build the calibration curve. As for many of the biosensors
548 described in the revised papers, the protocol for the fabrication of the electrode is not friendly,
549 and authors reported in the conclusions that the investigation of a simplified procedure was
550 ongoing.

551 **5.3 DNA**

552 Saini et al. developed a DNA-based biosensor to detect *Salmonella* Enterica in raw milk samples
553 [96]. The ssDNA probe was immobilized on a commercial screen-printed carboxylated
554 MWCNTs/AuNPs electrode, and the quantitative determination in milk samples was carried out
555 using CV. The authors declared a LOD of 0.3 pg/ml and the calibration curve showed a hyperbolic
556 relationship both for CV and DPV. The sensor gave a "presence/absence" response when used
557 to detect the bacteria in raw and spiked milk samples in 30 minutes. If stored at 4°C and regularly
558 used every 30 days, it proved to be stable during a period of 6 months with only 10% loss in the
559 initial intensity value.

560

561 **6. Other electrochemical biosensors**

562 Most of the reported biosensors for the detection of pathogenic bacteria in food are
563 impedimetric or voltammetric biosensors, but other types of electrochemical biosensors have
564 also been reported. The reviewed manuscripts in this section are summarized in Table 3.

565 Silva et al. [97] developed a disposable potentiometric paper-based biosensor for the detection
566 of S. Thyphimurium. Antibodies for the target bacteria were covalently attached to filter paper
567 coated with PEDOT:PSS via carbodiimide chemistry. A linear range from $12\text{-}12\cdot10^3$ CFU/ml with
568 a limit of detection of 5 CFU/ml were reported. The time of analysis was less than 5 minutes. S.
569 Thyphimurium was added to apple juice samples (pre-treatment includes a filtration process)
570 and mean recovery values of 54% were reported. No selectivity studies were reported. The same
571 research group developed another potentiometric biosensor conjugating S. Thyphimurium

572 antibodies on a gold nanoparticle polymer inclusion membrane in a home-made pipette tip [98].
573 Performance parameters were similar to their previously reported biosensor.

574 Son et al. [99] developed a carbon nanotube field-effect transistor (CNT-FET) [100] for the
575 indirect detection of *Salmonella*. The CNT-FET was fabricated by photolithography over SiO_2
576 substrates and carbon nanotubes were incubated from solution (no information about the type
577 of carbon nanotubes was given). A *Drosophila* odorant binding protein was immobilized over
578 carbon nanotubes by π-π interactions. Since this peptide has a specific alcohol-binding site, the
579 CNT-FET is able detect the 3-methyl-1-butanol that is generated upon contamination of ham by
580 *Salmonella* in an analysis time of 2 seconds. *Salmonella* was spiked in ham samples and the
581 generated signal was significantly higher than the signal corresponding to uncontaminated ham.
582 No minimum amount of *Salmonella* that can be detected was reported, so the biosensor remains
583 for qualitative results. Selectivity was checked against other odorants but not contaminating real
584 samples with other bacteria.

585 A chemiresistor for the detection *Salmonella* was developed by Thiha et al. [101]. In their device,
586 involving several steps of photolithography, SU-8 photoresist nanofibers were electrospun over
587 the supporting structures. Carbon nanowires were formed upon pyrolysis of the SU-8 fibers, and
588 were then functionalized with the same aptamer [54] extensively used in other previously
589 reported electrochemical biosensors (Figure 3). The biosensor was then integrated with a
590 microfluidic chip to form a compact lab-on-a-chip device. The calibration line seems to be built
591 (not clearly reported) between 10^1-10^8 CFU/ml, although clear non-linearities at high
592 concentrations can be observed. The reported limit of detection is 10 CFU/ml in 5 minutes of
593 analysis. S. Thyphimurium was spiked to beef samples (homogenized and digested for 4 hours)
594 and recovery studies ranged between 55%-167%.

595 The last type of reviewed biosensors are capacitive biosensors [102], in which changes in the
596 electrical capacitance of the biosensor due to binding processes involving the target analyte are
597 monitored. Idil et al. [103] immobilized *E. coli* over a glass slide, and this glass slide was then
598 used as a stamp to create suitable recognition cavities over a gold microelectrode coated with a
599 monomer mixture. Further polymerization under UV light created the MIP over the surface of
600 the gold electrode. The calibration range was reported between 10^2-10^7 CFU/ml with a limit of
601 detection of 70 CFU/ml and a time of analysis of about 30 minutes. Selectivity was rigorously
602 calculated with selectivity coefficients. Apple juice samples (diluted 1:10) were spiked with *E.*
603 *coli*. No recovery values were presented, only qualitative results showing the change in the
604 instrumental response. The device can be reused at least 70 times, with a regeneration time of
605 45 minutes.

606 Niyomdecha et al. [104] reported the development of a capacitive biosensor coupled to a flow
607 injection system using a *Salmonella*-specific bacteriophage for *Salmonella* detection. The
608 bacteriophage was immobilized on a polytyramine/gold surface. The device was extensively
609 characterized for most of the performance parameters, including testing selectivity with eight
610 different strains of *Salmonella*. A linear range from $2 \cdot 10^2$-10^7 CFU/ml with a detection limit of
611 200 CFU/ml were reported in an analysis time of 40 minutes. Binding and regenerating cycles
612 were operated continuously in the flow injection system, and the device was able to give
613 repetitive results up to 40 times. Minced chicken meat samples (diluted and filtered) spiked with
614 *Salmonella* were used to assess the trueness of the results.

615

616 **7. Conclusions and prospects**

We can safely say that electrochemical biosensors for the detection of pathogenic bacteria in food are reaching scientific maturity. The past five years have seen the publication of a significant number of excellent scientific papers providing impressive results that were difficult to imagine only a couple of decades ago. Not a small number of papers report limits of detection below 10 CFU/ml in less than one hour of analysis (pre-treatments for complex food samples are not considered in this time of analysis). Linear ranges usually cover several orders of magnitude, recovery values (when reported) are usually close to 100% (typical errors for all types of reviewed biosensors are around 10%) and selectivity is checked with different bacteria, even including different strains of the target pathogen. Precision is not always checked, but when precision is reported repeatability values are usually around 5% and intermediate precision values are also good. The emergence of these excellent results is probably explained by several factors. On one hand, in the last years we have seen the introduction of a plethora of new materials, like nanostructured-materials [31] with excellent properties for sensing such as very large surface-to-volume ratios or outstanding electrical properties. On the other hand, the introduction of efficient techniques of chemical and biochemical amplification [105]. The increased use of low-cost materials [106], the emergence of printed electronics as an alternative to silicon technology for the production of very low-cost electronic devices [107] or recent advances in screen-printing technologies [108] linked to mass-production are also reasons to expect a significant decrease in the cost of these devices and the progressive introduction of disposable single-use devices avoiding therefore cross-contamination of samples. Regarding the type of tested bacteria, most of the manuscripts report biosensors for *Escherichia* or *Salmonella*, what is consistent with major responsible bacteria in reported foodborne outbreaks [3,109]. But there is a lack of reported biosensors for bacteria such as *Campylobacter jejuni* and *Clostridium perfringens*, probably because of the lack of suitable receptors for the construction of the biosensor.

There are still some areas for improvement. Distinction between viable and dead cells is not always reported. Calculating recovery values with spiked food samples is a common way to assess the accuracy but it should ideally be assessed making a rigorous statistical comparison (including different concentrations of bacteria) with a well-stablished technique such as ELISA or PCR. All the papers reviewed in this manuscript report applications to food samples, but the majority of the samples tested are liquid samples such as milk, fruit juices or broths, used as a proof-of-concept. Little or nothing is known about the application of the described biosensors to complex food that can result in serious interferences from food matrices [11]. Other information, such as clearly specifying the time of analysis or whether the sample is pre-treated or not, should also be included in the articles.

Experimental design is also very rarely used in the development of the biosensor. Authors study the variables influencing the construction and the performance of the biosensors with a classical “one variable at a time” approach. With this approach, the interactions between variables or nonlinear behaviours are not considered, and therefore the optimum operation conditions, both in the construction and in the use of the biosensor, might not be found.

Focussing on the performance parameters, it is difficult to make a rigorous comparison among the reported limits of detection because many papers simply consider the limit of detection as the lower limit of the linear range. Similarly, the limit of quantification is rarely calculated. It would be also important to regularly include in the manuscripts information about the precision of the biosensor (repeatability and intermediate precision).

662 Another field of improvement is multiplexing sensing. While simultaneous electrochemical
663 detection of multiple analytes is a common solution in many fields, only a few biosensors for
664 multiplexing detection are reported. Typical foodborne outbreaks are usually caused by a single
665 strain of bacteria, but food quality control often requires the assessment of different bacteria
666 for a same product (for instance, control of *Campylobacter* spp., S. Thyphimurium and S.
667 Enteritidis in poultry samples). In this area, multiplexed biosensors could significantly help food
668 industry in maintaining or improving their quality standards.

669 Globally speaking, most of the reported biosensors in the scientific literature provide excellent
670 performance parameters, and some of them could take the leap towards commercialization
671 probably inspired by the undisputed success of glucose biosensors (85% of the world biosensor
672 market [26] with a prediction of USD 31.0 billion by 2022 [110]). This success has been tried to
673 reproduce with similar analytes [111] and there is considerable talk of biosensors being
674 introduced into daily life in the near future. But commercialization requires completely
675 integrated systems, and some drawbacks that hinder the widespread use of these devices are
676 the need for complete validation of biosensors and the application to food samples with minimal
677 sample pre-treatment. Even liquid samples such as milk are usually pre-treated (e.g.
678 centrifugation and/or filtration), so probably biosensors should also go hand-in-hand with more
679 automated (to be used in industrial food control applications) or portable pre-treatment
680 systems (to be used in on-site analysis). Without these systems of pre-treatment, biosensors
681 may lose the ability to be used on-site or in industrial food control applications and remain as
682 excellent alternatives to conventional methods of analysis in the laboratory.

683 Another important factor for commercialization is assessing the reproducibility in the
684 construction of the devices. Studies about the stability of the device or regeneration studies to
685 establish the number of times the biosensor can be re-used (or used only once if it is a disposable
686 device) should also be done and reported.

687 Finally, for a real generalised use, biosensors need to be user-friendly and usable by non-skilled
688 personnel with the potential to be handled as stand-alone devices for on-site monitoring. The
689 fact that most of the innovation in biosensors is led by people in universities and start-ups [26],
690 which do not have the financing muscle or workforce capacity of large companies, also hinders
691 the widespread commercialization of these biosensors and is probably the reason of the reduced
692 availability of commercial devices [112] for the detection of pathogenic bacteria in food.
693 Nevertheless, nowadays regulations with zero-tolerance [113] in certain foods, the increasing
694 need for point-of-care methods of analysis [114] and the growing consumer demand for safe
695 and healthy food will probably push the industry to develop faster and on-site methods of
696 analysis, where biosensors may play an important role. It is important to note that the
697 development of portable electrochemical instrumentation [115], using smartphones as portable
698 interfaces [116,117] could facilitate the commercialization of electrochemistry-based
699 biosensors.

700

701 **8. Abbreviations**

702 Ab: antibody
703 Apt: aptamer
704 CHI: chitosan
705 cMWCNT: carboxylated multi-walled
706 carbon nanotubes
707 CNT: carbon nanotube
708 CPE: carbon paste electrode
709 CV: Cyclic voltammetry
710 DPV: Differential pulse voltammery

EIS: Electrochemical impedance spectroscopy
Cys: cysteine
Exo III: exonuclease III
FCA: ferrocenecarboxylic acid
GCE: glassy carbon electrode
GO: graphene oxide
HMS: hierarchical mesoporous silica
HRP: horseradish peroxidase
ITO: indium tin oxide
MIP: molecularly imprinted polymer
MWCNT: multi-walled carbon nanotubes
NP: nanoparticle
NPG: nanoporous gold
NR: neutral red
PABA: poly(p-aminobenzoic acid)
PAMAM(Au): dendrimer-encapsulated gold nanoparticles
PANI: polyaniline
PBS: phosphate-buffered solution
PDDA: poly(diallyldimethylammonium chloride)
PEDOT: poly(3,4-ethylenedioxythiophene)
PEDOT:PSS: PEDOT:PSS or poly(3,4-ethylenedioxythiophene) polystyrene sulfonate
PEI: polyethylenimine
PGE: pencil graphite electrode
PIM: polymer inclusion membrane
PPy: polypirrole
RCA: rolling circle amplification
rGO: reduced graphene oxide
SpA: protein A
SPE: screen printed electrode
SWCNT: single-walled carbon nanotubes
ZIF-8: zeolitic imidazolate framework-8

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1135

1136 **Figure captions**

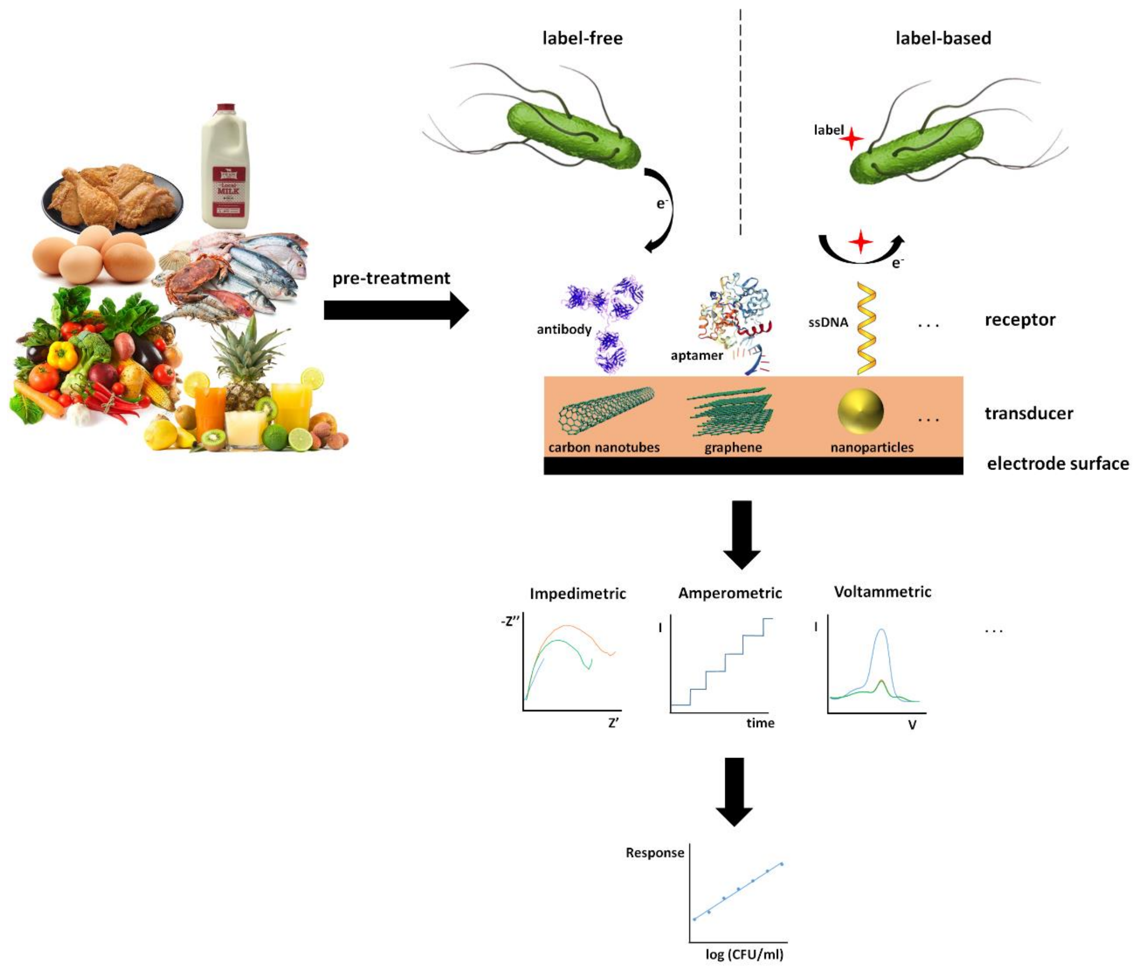
1137 Figure 1. Schematic of a biosensor showing the transducer in close contact with the receptor.

1138 Figure 2. a) Microfluidic-based impedance biosensor b) Schematic of the testing setup.
1139 Reproduced with permission from [47].

1140 Figure 3. Schematic of the sandwich assay for the labelled detection of *E. coli* O157:H7. The
1141 immobilized antibody over GCE/sulfonated graphene/PEDOT-Au captures the target bacteria. A
1142 HRP-labelled antibody attaches to the captured analyte and the HRP produces the measurable
1143 electrochemical signal. Reproduced with permission from [72].

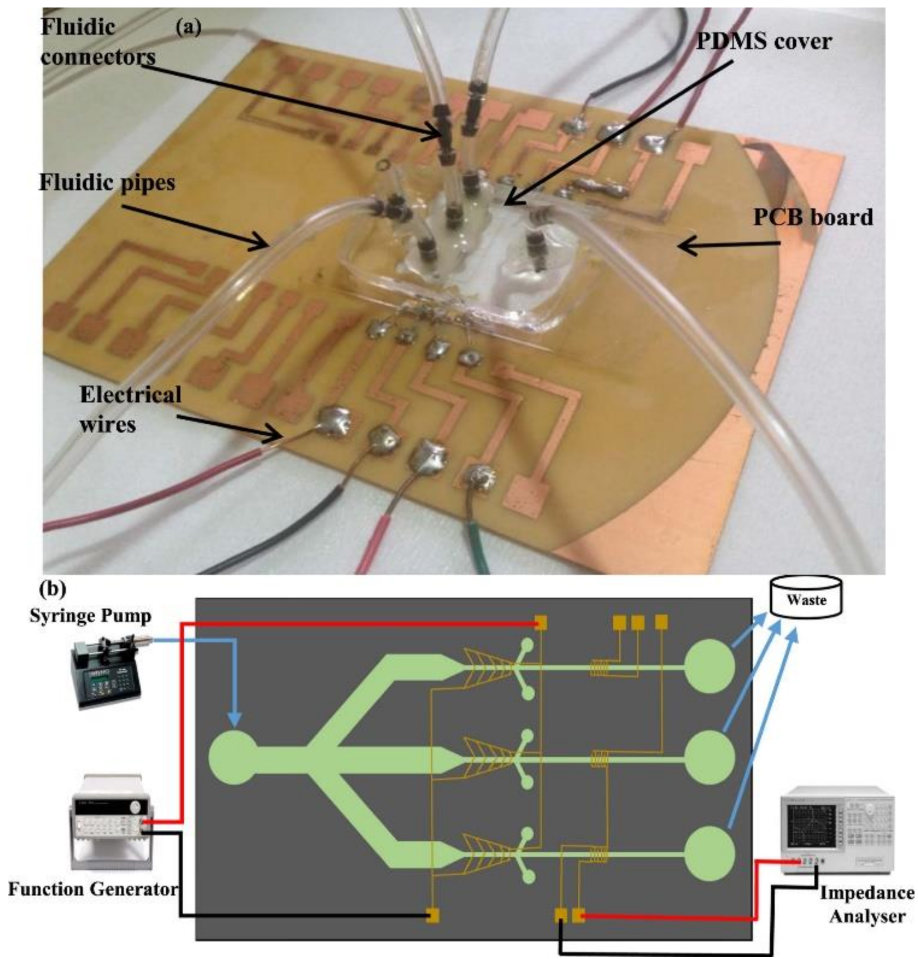
1144 Figure 4. Fabrication of the carbon nanowire sensor chip, from electrospun carbon nanowires
1145 (a) to the final microfluidic platform (h). Reproduced with permission from [101].

1146 **Figure 1**



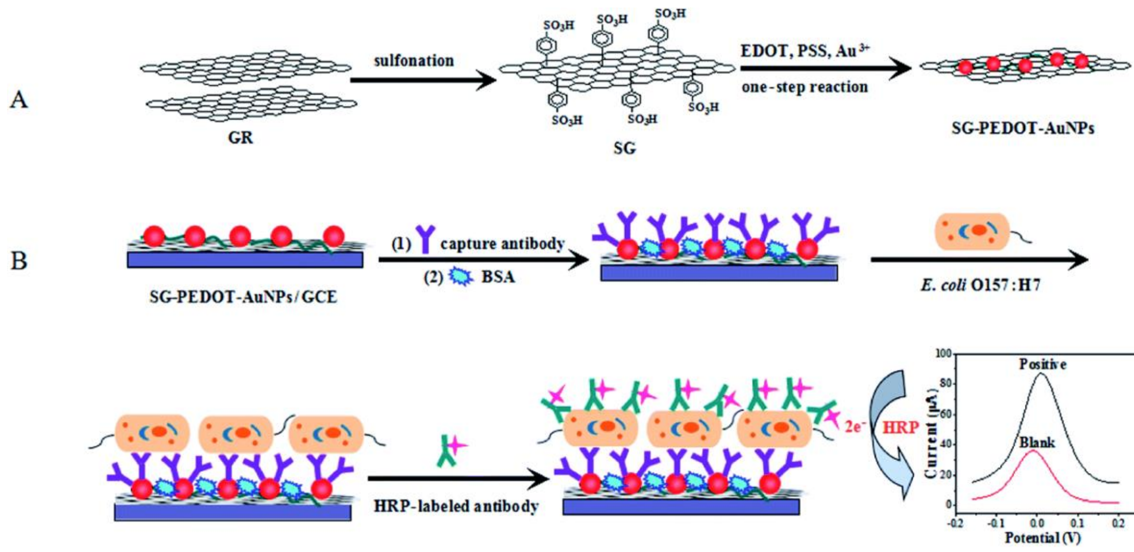
1147

1148 **Figure 2**



1149

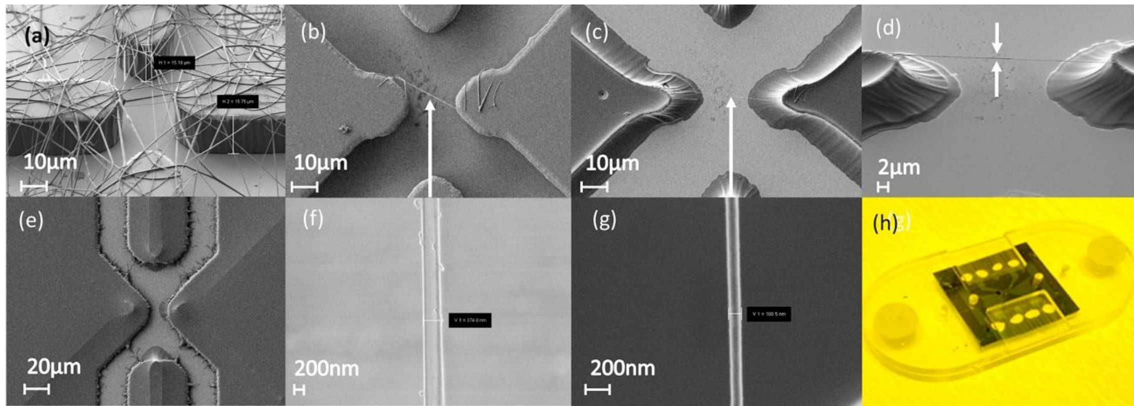
1150 **Figure 3**



1151

1152 **Figure 4**

1153



1154 Table 1. Summary of the reviewed impedimetric biosensors. Biosensors are divided according to the receptor used

1155

Target pathogen	Sensing strategy	Label	Linear range LOD (CFU/ml)	Assay time	Application to real samples	Pre-treatment to real samples	Paper
E. coli **O157:H7**	Au/Cys/Ferrocene/Ab	no	depending on the strategy	90 min	milk and meat	meat (digested and filtered), milk (1:10 diluted in PBS)	[45]
E. coli **K12**	Au/Ab	no	10^3-10^5 10^3	not reported	frozen chicken	not reported	[46]
Salmonella	Cr-Au/Ab	no	not defined 7	40 min	poultry products	adding peptone water + enrichment + filtering + addition glycerol	[47]
S. Typhimurium	C SPE/rGO/Ab	no	10^2-10^6 10	10 min	fruit juice	not reported	[48]
S. Typhimurium	Au SPE/Ab	no	10^5-10^8 $7 \cdot 10^4$	20 min	milk	mild shaking	[49]
S. Typhimurium	Au/Ab	no	not reported	1 h	ready-to-eat turkey	filtration through a cell strainer	[50]

Listeria monocytogenes* and *Staphylococcus aureus	Au/Ab	no	depending on the bacterium	1 h	pork meat sausage	digestion	[51]
Listeria monocytogenes	Cr-Au/Ab	no	depending on the matrix	1 h	milk	dilution (4:1)	[52]
***Salmonella* enterica**	GCE/rGO/cMWCNTs/Apt	no	$7.5 \cdot 10^1$-$7.5 \cdot 10^5$ 25	60 min	chicken samples	addition of normal saline and stirring	[55]
S. Typhimurium	C SPE/Diazonium/Apt	no	10^1-10^8 6	45 min	apple juice	not reported	[56]
S. Typhimurium	Au/poly[pyrrole-co-3-carboxyl-pyrrole] copolymer/Apt	no	10^2-10^8 3	45 min	apple juice	dilution	[57]
S. Typhimurium	GCE/Au/NPG/Apt	no	$6.5 \cdot 10^2$-$6.5 \cdot 10^8$ 1	40 min	egg samples	centrifugation and dilution	[58]
***Salmonella* enterica**	ITO/cMWCNTs/Apt	no	$6.7 \cdot 10^1$-$6.7 \cdot 10^5$ 67	20 min	chicken samples	incubation 3 hours, overnight enrichment, centrifugation	[59]
S. Typhimurium	SPE/AuNPs/Apt	no	10^1-10^5 10	not reported	whole liquid egg	mixing, homogenization and dilution	[60]

Listeria monocytogenes	nPt/rGO/CHI nanobrush/Apt	no	10^{0}-10^{7} 3	17 min	vegetal broth	not required	[61]
Shigella dysenteriae	GCE/AuNP/Apt	no	10^{1}-10^{8} 10	30 min	milk	diluted milk (1:10)	[62]
Bacillus cereus	PGE/AuNP/ssDNA	no	not reported for bacteria 10 CFU/ml	estimated 2-3 h	milk and infant formula	DNA extraction	[63]
Clostridium perfringens	GCE/ CHI-CeO_2 nanorods/ssDNA	no	not reported for bacteria	30 min	milk	ultrasonic extraction, centrifugation	[64]
E. *coli* O157:H7	GCE/MIP	no	10^{3}-10^{8} 3	1 h	apple juice and milk	apple juice: 1:10 dilution milk: centrifugation, filtration, dilution	[65]
Staphylococcus arlettae	carboxilated graphene SPE/ Bacteriophage	no	2-$2\cdot10^{6}$ 2	2 min	apple juice	filtration and resuspension	[67]

1156

1157

1158 Table 2. Summary of the reviewed amperometric/voltammetric biosensors. Biosensors are divided according to the receptor used

Target pathogen	Sensing strategy	Label	Linear range LOD (CFU/ml)	Assay time	Application to real samples	Pre-treatment to real samples	Paper
***E. coli* O157:H7**	ITO/NiO/Ab	no	10^{1}-10^{7} 10	1h incubation, 5 sec analysis	diluted milk	not reported	[70][1]
***E. coli* O157:H7**	CdS@ZIF-8@PEI-Ab Ab-PABA/GCE	yes	10^{1}-10^{8} 3	about 2 h	diluted milk	dilution 1:10 with PBS	[71][2]
***E. coli* O157:H7**	GCE/sulfonated graphene/PEDOT-AuNPs/Ab Ab-HRP	yes	$7.8\cdot10^{1}$-$7.8\cdot10^{6}$ 3.4	2.5 h	diluted milk	dilution 1:10 with PBS	[72] [2]
***E. coli* K12**	GCE/PPy-rGO/AuNPs/Ab1 Ab2/AuNPs/PPy@Ferrocene	yes	10^{1}-10^{7} 10	2 h	milk	not reported	[73] [2]
***E. coli* O157:H7**	C SPE/PANI/AuNPs/Ab1 Ab2/Au@Pt/rGO-NR	yes	$8.9\cdot10^{3}$-$8.9\cdot10^{9}$ $2.84\cdot10^{3}$	60 min	milk powder and pork samples	beated (meat) and dissolved in PBS	[74][1]
E. coli	Au/Ab Ab/Au nanorods/FCA	yes	$1\cdot10^{2}$-$5\cdot10^{4}$ 60	3 h	milk and yogurt	dilution 1:10 PBS	[75][2]

E. coli	GCE/CHI/PAMAM(Au)/Ab Ab/MWCNT-HRP	yes	$1\cdot10^{2}$-$1\cdot10^{6}$ 50	3 h	milk and yogurt	dilution 1:10 PBS	[76][2]
E. coli	GCE/GO/PDDA/AuNP/Ab Ab/AuNP	yes	$5\cdot10^{1}$-$5\cdot10^{6}$ 35	about 2 h	milk and yogurt	dilution 1:10 PBS	[77][2]
E. coli	Au/Ab Apt/circular probe/hemin	no	$9.4\cdot10^{1}$-$9.4\cdot10^{5}$ 8	less than 2.5 h	milk	dilution	[78][2]
Salmonella	GCE/Chi-AuNPs/Ab Ab-HRP	yes	10^{1}-10^{5} 5	4h (fabric and analysis time)	milk	not reported	[80][2]
Salmonella pullorum* and *Salmonella gallinarum	C SPE/AuNPs/Ab Ab-HRP	yes	10^{4}-10^{9} $3\cdot10^{3}$	50 min	eggs/chicken meat	not reported	[81][1]
S. Typhimurium	Au/Cys/SpA/Ab Ab-HRP	yes	not reported 10	125 min (155 minutes for whole milk)	skimmed and whole milk	sample centrifugation for whole milk	[82][3]
S. Typhimurium	GCE/CHI/AuNPs/Ab/AuNPs-HRP/CHI/Ab	yes	10^{1}-$5\cdot10^{4}$ 5	15 min	milk	adjust pH to 7.2-7.4	[83][4]
Staphylococcus aureus	GCE/HMS/Ab	no	10^{1}-$2\cdot10^{3}$ 11	20 min	whole milk	not reported	[84][2]

Staphylococcus aureus	CPE/SWCNTs/Ab	no	10^{1}-10^{7} 13	30 min	milk	20% v/v in PBS	[85][2]
Lysteria monocytogenes	MWCNT fibers/Ab-HRP	yes	10^{2}-10^{5} $1.7 \cdot 10^{2}$	30 min	milk	dilution (1:10)	[86][1]
E. coli	Au/Apt/Apt	no	$5 \cdot 10^{2}$-$5 \cdot 10^{7}$ 80	150 min	licorice extract	diluted licorice extracts with solid content of 4%	[87][2]
***Salmonella* enterica**	GCE/rGO-CHI/Apt	no	10^{1}-$5 \cdot 10^{6}$ 10	about 2 h	raw chicken	homogenization and dilution	[88][2]
S. Typhimurium	CGE/rGO-azophloxine/Apt	no	10^{1}-10^{8} 10	10 min	raw chicken	homogenization and dilution	[89][2]
S. Typhimurium	Au/AuNPs/Apt/RCA	yes	20-$2 \cdot 10^{8}$ 18	3h	milk	not reported	[90][2]
Salmonella	Au/Apt/AuNPs/streptavin-alkaline phosphatase	yes	$2 \cdot 10^{1}$-$2 \cdot 10^{6}$ 20	not reported	milk	not reported	[91][2]
S. Typhimurium	GCE/graphene/ UiO-67/Apt-HRP	yes	$2 \cdot 10^{1}$-$2 \cdot 10^{8}$ 5	3 h	purified milk	not reported	[92][2]

S. Typhimurium	Au/ssDNA/Exo III-aided autonomous cascade signal amplification	yes	$7.2\cdot10^{1}$-$7.2\cdot10^{6}$ 28	110 min	milk	dilution with buffer	[93][2]
S. Typhimurium	Au/ssDNA/Exo III-assisted autonomous multiple-cycle amplification	yes	$1\cdot10^{1}$-$1\cdot10^{6}$ 8	4 h	milk	1:4 dilution PBS	[94][2]
Vibrio parahaemolyticus	Au/Ab Apt/AuNPs/RCA	yes	$2.2\cdot10^{1}$-$2.2\cdot10^{8}$ 2	1-2 h	spiked fish samples	not reported	[95][2]
***Salmonella* enterica**	cMWCNT/AuNPs/ssDNA	no	0.3 pg/ml	30 min	milk	none	[96][1]

1159 [1] Cyclic voltammetry, [2] Differential pulse voltammetry, [3] Chronoamperometry, [4] Amperometry

1160

1161 Table 3. Summary of other type of biosensors

Target pathogen	Sensing strategy	Label	Linear range LOD (CFU/ml)	Assay time	Application to real samples	Pre-treatment to real samples	Paper
S. Typhimurium	Paper/PEDOT:PSS/Ab	no	$12\text{-}12\cdot10^3$ 6	<60 min (including pre-treatments)	apple juice	filtration and washing	[97][1]
S. Typhimurium	Internal solution/AuNP-PIM/Ab	no	$13\text{-}13\cdot10^2$ 6	<60 min (including pre-treatments)	apple juice	filtration and washing	[98][1]
S. Typhimurium	SiO_2/octacyltrichlorosilane/CNT/Peptide	no	not reported not reported	2 sec	ham	addition of water and dilution	[99][2]
S. Typhimurium	C/Apt	no	$10^1\text{-}10^8$ 10	5 min analysis + 4 h pre-treatment	beef	homogenization and digestion	[101][3]
E. *coli*	Au/MIP	no	$10^2\text{-}10^7$ 70	around 30 min	apple juice	dilution (1:10)	[103][4]
***Salmonella* spp.**	Au/polytyramine/ Bacteriophage	no	$2\cdot10^2\text{-}10^7$ 200	40 min	minced chicken meat	dilution and filtering	[104][4]

1162 [1] Potentiometric, [2] Field-effect-transistor, [3] Chemiresistor, [4] Capacitive