Identification of colonies of cultured shellfish-associated *Arcobacter* species by Elastic Light Scatter Analysis

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ABSTRACT

An increasing number of *Arcobacter* species (including several regarded as emerging human foodborne pathogens) have been isolated from shellfish, an important food commodity. A method to distinguish these species and render viable isolates for further analysis would benefit epidemiological and ecological studies. We describe a method based on Elastic Light Scatter analysis (ELSA) for the detection and discrimination of eleven shellfish-associated *Arcobacter* species. Although substantive differences in the growth rates of some taxa were seen, ELSA was able to differentiate all the species studied, apart from some strains of *A. butzleri* and *A. cryaerophilus*, which were nonetheless distinguished from all other species examined. ELSA appears to be a promising new approach for the detection and identification of *Arcobacter* species in shellfish and may also be applicable for studies in other foods and matrices.

The genus *Arcobacter* represents a group of aerotolerant and somewhat psychrophilic bacteria, phylogenetically closely related to *Campylobacter* (On, Miller et al. 2020). Individual *Arcobacter* species may be free-living, having been described from sources including plant root nodules, and fresh- and seawater domains (McClung et al. 1983, Collado, Guarro et al. 2009, On, Althaus et al. 2019), while host-associated species have been associated with ovine, bovine and porcine reproductive diseases (Neill, Ellis et al. 1979, On, Jensen et al. 2002, De Smet 2011, Di Blasio, Traversa et al. 2019), as well as enteric diseases in humans (Vandamme, Pugina et al. 1992, Lerner, Brumberger et al. 1994, Vandenberg, Dediste et al. 2004, Wybo, Breynaert et al. 2004). The association of taxa, including *A. butzleri* and *A. cryaerophilus,* with foods of animal origin (Atabay, Corry et al. 1998, Atabay, Aydin et al. 2003, Andersen, Wesley et al. 2007, Aydin, Gumussoy et al. 2007, Duffy and Fegan 2012, Hsu and Lee 2015, Caruso, Latorre et al. 2018, Fanelli, Di Pinto et al. 2019, On, Althaus et al. 2019), as well as an increasing number of studies revealing their presence in cases of human gastroenteritis (Vandamme, Pugina et al. 1992, Lerner, Brumberger et al. 1994, Engberg, On et al. 2000, Vandenberg, Dediste et al. 2004, Wybo, Breynaert et al. 2004) led to (and continues to support) the decision of the International Commission on Microbiological

Food Safety to declare *Arcobacter*s as emerging foodborne pathogens (ICMSF 2002).

Of the 28 species validly named to date (On, Miller et al. 2020), many have been recovered from shellfish, an important food source for coastal peoples and indeed a high-value export commodity (Nieva-Echevarria, Martinez-Malaxetxebarria et al. 2013, Rathlavath, Kohli et al. 2017, Rathlavath, Kumar et al. 2017, Noto, Sciortino et al. 2018, On, Althaus et al. 2019). Certain shellfish-associated arcobacters have not yet been associated with human gastroenteritis but others, including *A. butzleri, A. cryaerophilus* and *A. skirrowii* have had long-standing associations with enteric disease (Vandamme, Pugina et al. 1992, Lerner, Brumberger et al. 1994, Engberg, On et al. 2000, Vandenberg, Dediste et al. 2004, Wybo, Breynaert et al. 2004). Management of common-source outbreaks requires the application of methods that can expeditiously detect the target organism (and discriminate it from benign species), and ideally render an isolate available for further epidemiological analysis. The taxonomic complexity of *Arcobacter* makes detection by molecular methods such as PCR problematic; furthermore, such molecular methods are unable to recover isolates for further study.

Elastic Light Scatter Analysis (ELSA) involves the interrogation of a bacterial colony using laser light and the subsequent detection of

the light scatter pattern that is a function of its cellular ultrastructure and colony organisation (Bae, Patsekin et al. 2012). It has been used previously to discriminate species including *Campylobacter, Vibrio* and *Yersinia* that are notable foodborne pathogens (Huff, Aroonnual et al. 2012, He, Reed et al. 2015, On 2021). ELSA requires a suitable growth medium free from blood cells, and the rather fastidious nature of a*rcobacter*s has often required the use of blood agar in conjunction with membrane filtration in order to recover these species from complex matrices such as food and faeces (Atabay and Corry 1997, Engberg, On et al. 2000, On, Jensen et al. 2002, On, Althaus et al. 2019).

In this paper we describe the development of an ELSA method that is able to detect and discriminate several *Arcobacter* species found in shellfish that represent emerging-pathogenic and free-living species.

Materials and methods

Strains. A total of 28 strains representing 11 *Arcobacter* species were examined. Ten strains of *A. butzleri* and nine strains of *A. cryaerophilus* were examined: these species are the most commonly reported arcobacters in human gastroenteritis (Engberg, On et al. 2000, Vandenberg, Dediste et al. 2004). The type strains of each of the following species were also examined: *A. aquimarinus, A. cibarius, A. ellisii, A. skirrowii, A. bivalviorum, A. defluvii, A. mytili, A. cloacae* and *A. venerupis.* All eleven species studied have been isolated from shellfish (Figueras, Levican et al. 2011, Levican, Collado et al. 2012, Levican, Collado et al. 2013, Levican, Rubio-Arcos et al. 2015, Laishram, Rathlavath et al. 2016, Rathlavath, Kohli et al. 2017, Rathlavath, Kumar et al. 2017, On, Althaus et al. 2019).

Media. Strains were cultured on 5% blood agar (TSA base [Oxoid Ltd., UK] inclusive of 2% w/v NaCl for the species *A. aquimarinus, A. bivalviorum, A. defluvii, A. mytili* and *A. venerupis*) for 2–3 days before making initial bacterial suspension of ca. 0.5 Macfarland standard density, thereafter preparing dilutions containing 100–1000 colony forming units / ml in peptone water. Fifty μl aliquots of these suspensions were used to spread-inoculate, as before (Bae, Ying et al. 2012), the medium used for ELSA profile determination. This medium was a modified Brain Heart Infusion agar (Difco, New Jersey, USA) with added 0.5% (w/v) agar Noble (Difco). Twenty-five ml of a filter-sterilised solution of $FeSO_4$ (0.25 g $FeSO_4$ in 25 ml distilled water, adjusted to pH 4 with HCl) was added to 975 ml of the molten, autoclaved agar cooled to 55 °C, and mixed together by gentle swirling. For halophilic species, an additional 1.5% (w/v) NaCl was added to the agar prior to autoclaving. Then, 35 ml of the molten agar was dispensed into rectangular sterile tissue culture plates (Omni Tray, VWR, Philadelphia, USA) and allowed to set, carefully ensuring air bubbles were not present.

Elastic Light Scatter Analysis (ELSA). The principles of Elastic Light Scatter profiling have been described before (Bae, Kim et al. 2016). A custom-built scanner, as described previously (Tang et al. 2014), was used. This version of the scanner included an automated incubator (Cytomat C2, Thermo-Heraeus, Massachussets, USA) (here set to 25 °C) adjacently coupled to a robotic feeder mechanism allowing agar plates to be analysed at operator-specified time intervals. Our study examined *Arcobacter* growth every two hours after an initial period of six hours, allowing for the lag growth phase. Plates were examined for up to 65 h.

Database construction and assessment. The ELS profiles of the strains studied were examined over the time series used for collection, and at the differing colony magnifications (1X, 2X, 4X) available to the system. Decisions were made on which magnification setting and which time point to use based on (i) the earliest time at which discernible ELS profiles with sufficient complexity could be obtained, and (ii) level of complexity of ELS spectra at given magnificatition settings, with preference given to profiles that were more informative on the basis that these would likely prove more useful for species-level identification. A database containing no fewer than 50 suitable ELSA profiles for each taxon was constructed, and the effective separation of each was estimated using cross-validation, as described before (Tang et al. 2014). In

brief, the data set for each taxon is randomly divided into a training subset against which testing subsets from itself, and the other taxa, are compared and this process is repeated 10 times, to yield a statistical assessment of the level of separation beween taxa on the basis of the ELS profile data. Optimal separation of taxa is achieved when homologous comparisons achieve results exceeding 90, and heterologous comparisons yield values of 10 or below (On et al., 2021) in the summarised cross-validation matrix.

Results

Arcobacter growth characteristics. All strains examined were able to grow on the BHI-$FeSO_4$ medium used for ELSA. The use of an automated system coupled with image analysis also allowed for colonies to be detected at regular intervals, revealing vastly different growth characteristics between species. For example, *A. aquimarinus* colonies were detectable after just 14 h incubation, while *A. venerupis* was detected only after 57 h. The earliest time at which colonies were detected is given in Table 1.

Elastic Light Scatter Analysis (ELSA). Optimal images for analysis of the *Arcobacter* species examined were obtained at differing times of incubation, relating to the growth rate for each species (see above and Table 1). The optimal time (or time range) at which colonies of each species yielded an image considered to be best suited for image analysis is given in Table 1.

All species examined yielded colonies with characteristic ELS profiles (Fig. 1) under appropriate magnification (Table 2). Certain strains of *A. butzleri* and *A. cryaerophilus* exhibited patterns similar to each other (Table 1, Fig. 1); thus, for constructing the identification database to assess taxonomic separation, colony images of these strains were included as a single novel type, *A. butzleri-cryaerophilus* Type 2. The resulting database demonstrated homologous cross-validation values well above the value of 90 (Table 2) that is regarded as the benchmark for suitable performance.

Discussion

The use of our automated plate-reading system provided some unique insights into the growth characteristics of the *Arcobacter* species studied. To our knowledge, this is the first study that has examined the growth dynamics of these organisms under laboratory conditions on solid growth media. An important caveat to consider is that the instrumentation used has limitations in its ability to detect colonies of a certain size (ca. 1 mm; Banada et al. 2009), and we have considered detections only where ELS profiles are discernible. In some cases, both the optimal time for imaging and the first time at which colonies are detected are the same for this reason. Nonetheless, our study suggests considerable differences in the mean generation times between species, as observed previously for a few taxa (Houf, Devriese et al. 2001). These differences did not appear to be related to source or host, where known. The fastest (*A. aquimarinus*) and slowest (*A. venerupis*) growing species were both first recovered from shellfish (Levican, Collado et al. 2012, Levican, Rubio-Arcos et al. 2015) and the species studied (*A. butzleri, A. cryaerophilus, A. skirrowii)* that have been recovered from mammalian hosts such as cattle, sheep, and pigs as well as shellfish demonstrated considerable variation in their growth rates. This observation illustrates the challenges faced in clinical and food laboratories in diagnosing *Arcobacter* infections and the need for extended incubation times where culture-based methods are used, notably where slower-growing species are implicated. Clearly, similar challenges are faced where arcobacters are searched for in environmental samples. In both cases, the use of an automated system such as that used here holds certain advantages, where plate media can be examined at regular intervals and different taxa can also be recognised by their distinctive Elastic Light Scatter (ELS) profile.

Table 1

Arcobacter species and strains examined, and earliest time of detection and optimal time for Elastic Light Scatter Analysis (ELSA).

Arcobacter species	Strain number	Earliest time (hours) of detection by automated ELSA	Optimal time (hours) for imaging by ELSA
A. aquimarinus	LMG 27923[T]	14	18
A. bivalviorum	LMG 26154[T]	28	28
A. butzleri	RM4463*, RM4473*, RM4591*, RM4839*, RM4844, RM5214, RM5222*, RM5232, RM5548, RM5564	25.5	25.5–28
A. cryaerophilus	RM4491, RM4598, RM4616, RM4826*, RM5340, RM5342, RM5557*, RM5588, RM6740	28	28–42
A. cibarius	LMG 21996[T]	18	25.5
A. cloacae	LMG 26153[T]	18	21.5
A. defluvii	LMG 25694[T]	16	22
A. ellisii	LMG 26155[T]	47	47–48.5
A. mytili	LMG 24559[T]	18	33
A. skirrowii	LMG 6621[T]	26.5	49.5
A. venerupis	LMG 26156[T]	57	63.5

*These strains exhibited similar patterns and were assigned to the joint ELS type 2 (cf. Table 2).

Table 2

Cross-validation matrix of *Arcobacter* taxa examined by ELSA.

Taxon (magnification)	1	2	3	4	5	6	7	8	9	10	11	12
A. aquimarinus (1X)	100	0	0	0	0	0	0	0	0	0	0	0
A. butzleri Type1 (2X)	0	99	1	0	0	0	0	0	0	0	0	0
A. cryaerophilus Type 1 (2X)	0	0	97.5	2.5	0.1	0	0	0	0	0	0	0
A. butzleri-cryaerophilus Type 2 (2X)	0	0	2.6	96.9	0.3	0	0	0.2	0	0	0	0
A. skirrowii (2X)	2.2	0	0	5.3	92.2	0	0	0	0	0	0	0.2
A. bivalviorum (1X)	0	0	0	0	0	97.6	1.3	0	0	0	1	0
A. defluvii (1X)	0	0	0	0	0	5.1	93.2	1.8	0	0	0	0
A. mytili (1X)	0	0	0	2.2	0	0	0	96.9	0	0.9	0	0
A. venerupis (1X)	0	0	0	0	0	1.4	0	0	96	2.6	0	0
A. cibarius (1X)	0	0	0	0	0	0	0	1.5	2.4	96.1	0	0
A. cloacae (1X)	0	0	0	0	0	1.3	0	1.1	0.6	0	95.2	1.9
A. ellisii (1X)	0	0	0	0	0	0	6.7	0.2	0	0	0	93.1

Taxon codes: 1, *A. aquimarinus*; 2, *A. butzleri* Type1; 3, *A. cryaerophilus* Type 1; 4, *A. butzleri-cryaerophilus* Type 2; 5, *A. skirrowii*; 6, *A. bivalviorum*; 7, *A. defluvii*; 8, *A. mytili*; 9, *A. venerupis*; 10, *A. cibarius*; 11, *A. cloacae*; 12, *A. ellisii*.

In common with other studies on different organisms (Huff, Aroonnual et al. 2012, He, Reed et al. 2015), we found that most *Arcobacter* species yielded distinctive ELS profiles when examined (Fig. 1). The different growth rates exhibited however presented some particular challenges not described previously, as did some aspects of the colony analysis, whereby for some species (*A. butzleri, A. cryaerophilus, A. skirrowii*), a different magnification scale was required for acquisition of the best quality ELS image for analysis. This observation could not be attributed to growth rate alone, given the difference observed in this feature across all taxa (see above), and may be due to some other ultrastructural feature of the colonies of these species. Such features will include the size, shape and cell surface features (eg. flagella, cell wall composition) of individual bacterial cells, since all of these contribute to the structure of the cultured colony, ultimately influencing the eventual scatter pattern. We also determined that some strains of the established emerging pathogens *A. butzleri* and *A. cryaerophilus* yielded ELS profiles that were highly similar to each other (Table 1, Fig. 1). For best resolution, these profiles were considered together in a single taxonomic grouping, with the subsequent cross-validation analysis (Table 2) demonstrating excellent performance characteristics on this basis. Although we cannot at present claim unequivocal discrimination of *A. butzleri* from *A. cryaerophilus,* this orientation of the database does seem to enable the established emerging pathogenic species (including the latter two mentioned) to be differentiated from those that have thus far not been associated with any form of human illness. In summary, the use of an automated ELS system has provided novel insights into the growth rates of 11 shellfish-associated *Arcobacter* species of environmental and/or public health significance. The method also demonstrates considerable

potential for studies of the taxonomic diversity of arcobacters where isolates are required for further study, and offers a unique approach for discriminating emerging pathogenic species from those that are free-living. Nevertheless, additional work is required, with more isolates, to investigate the potential of ELSA to discriminate a wider number of *Arcobacter* species, and possibly the phylogenetically related *Campylobacter* and *Helicobacter* species also, since many of these organisms have also been associated with foodborne disease (Engberg *et al.* 2000, ICMSF 2002). Use of ELSA as an adjunctive detection method for arcobacters, potentially in conjunction with other isolation media that has been shown to enhance the recovery of marine species (Rahman et al. 2020), would also be of interest, given recent results for other foodborne pathogens (On et al. 2021). Thus, further studies into the application of ELSA for detection and identification of a wider range of *Arcobacter* and related species found in other matrices are warranted.
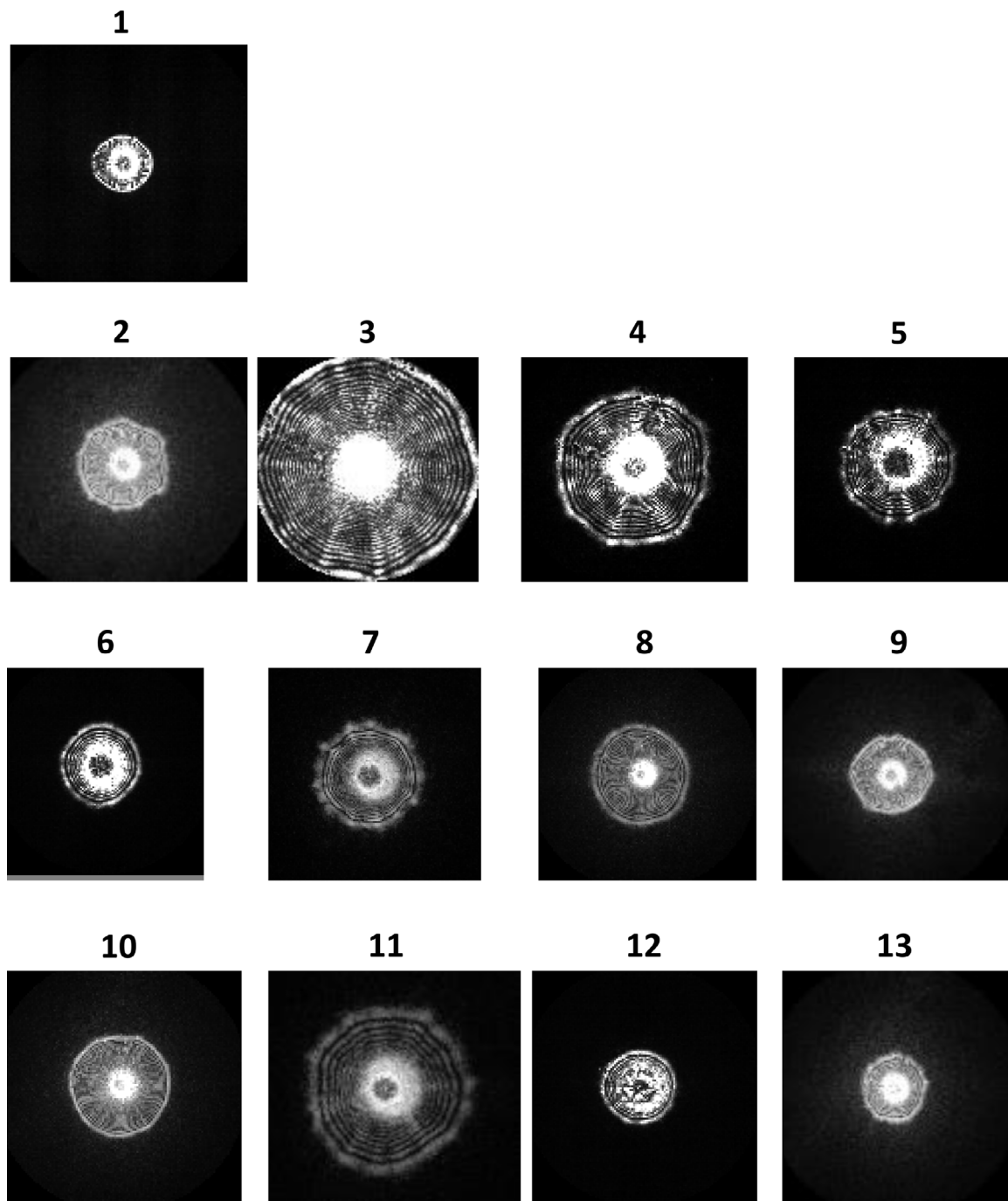
Author Contributions

Fig. 1. Representative Elastic Light Scatter profiles of *Arcobacter* species examined. 1, *A. aquimarinus*; 2, *A. bivalviorum*; 3, *A. butzleri* ELS 1 RM 5548; 4, *A. cryaerophilus* ELS 1 RM 6740; 5, *A. cryaerophilus* ELS 2 RM 4463; 6, *A. butzleri* ELS 2 RM 5557; 7, *A. cibarius*; 8, *A. cloacae*; 9, *A. defluvii*; 10, *A. ellisii*; 11, *A. mytili*; 12, *A. skirrowii*; 13, *A. venerupis*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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