Fish photobacteriosis—The importance of rapid and accurate identification of Photobacterium damselae subsp. piscicida

Full title: Fish photobacteriosis – the importance of rapid and accurate identification of *Photobacterium damselae* subsp. *piscicida*

Running title: **MALDI-TOF MS for *Ph. damselae* subsp. *piscicida***

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ABSTRACT

MALDI-TOF MS was tested for identification of *Photobacterium damselae* subsp. *piscicida* on isolates grown on two media, cultured at three incubation times, applied on the target plate by the direct sample spotting (DS), the on-target extraction (OTE) and by the full extraction (FE) method, in triplicates. Identification of samples grown on blood agar (BA) outperformed identification on tryptic soy agar (TSA) by 0.64% for DS and OTE. The OTE gave highest scores in both culture media, all incubation times and replicates. Reliable 24 h species identification were 61.54 %, 84.61 %, and 53.85 % for samples grown on TSA and identified by DS, OTE and FE, respectively. For isolates grown on BA they were 76.92 %, 96.15 %, and 30.77 %, respectively. When identified by OTE, the 48 h identification was 93.58 %, but for 72 h declined to 71.79 %. The reliable identification with the highest score from the first measurement was 100 % only for OTE from BA (24 h), whereas OTE from TSA gave 84.61 % (24 h), 76.92 % (48 h), and 84.61 % (72 h). The reliable MALDI-TOF MS identification of *Ph. damselae* subsp. *piscicida* is incubation time, media, target plate preparation, and replicate dependent.

KEYWORDS

Photobacterium damselae subsp. *piscicida*, MALDI-TOF MS

1. INTRODUCTION

Photobacterium damselae subsp. *piscicida* is a bacterium causing a serious health condition of both farmed and wild fish populations. It gives rise to a septicaemia with only few signs in the acute phase. Typical skin lesions are manifested as granulomatous ulcerative dermatitis, particularly in the region of the pectoral fin and caudal peduncle. Internally, granulomatous-like deposits may occur on liver, kidney and spleen. The condition was termed pasteurellosis, photobacteriosis and/or pseudotuberculosis due to the distinctive pathology (Austin and Austin, 2007). The disease has been responsible for heavy losses in farmed marine fish worldwide, and has also spread to farmed and wild fish stocks in the Mediterranean area (Toranzo et al., 1991; Magariños et al., 2003; Mladineo et al., 2006).

The organism may be isolated by inoculating swabs of kidney and/or spleen material onto marine agar 2216E, tryptic soy agar (TSA), nutrient agar or blood agar supplemented with 1-2% NaCl, with incubation at 22-25°C for 48–72 h. On conventional media, shiny, grey-yellow, entire, convex colonies develop (Romalde, 2002; Austin and Austin, 2007). It may be identified by phenotypic methods such as conventional plate and tube tests, by modified API 20E rapid identification system (Topić Popović et al., 2007) with a typical profile number 2005004 (Romalde, 2002). It can also be identified by serological analysis with slide agglutination tests (Magariños et al., 1992), and with ELISA tests (Bakopoulos et al., 1997). Various molecular tools were applied for its identification and differentiation from the subspecies *damselae*, including multiplex-PCR, AFLP or PCR-RFLP assays (Osorio et al., 1999; Osorio et al., 2000; Kvitt et al., 2002, Zappulli et al., 2005; Amagliani et al., 2009).

Although some of the tests listed above are straightforward and highly reproducible in identification of *Ph. damselae* subsp. *piscicida*, the complete identification frequently is time consuming, some methods require specialized training, technical skills and support, often are

labour-intensive, while some require enrichment of target organism and costly reagents (Topić Popović et al., 2017). Nevertheless, *Ph. damselae* subsp. *piscicida* outbreaks in fish require fast and reliable identification in order to move to the next step of prescribing treatment and control measures. To that effect, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is the most promising method for bacterial identification. It is a rapid and accurate proteomic method, detecting ribosomal protein fractions of bacteria to be used for classification of the organisms (Lay, 2001). Further identification is based on the detection of mass signals from proteins specific at genus, species or subspecies levels (Benagli et al., 2012). MALDI-TOF MS analyses whole bacterial cells within minutes after cultivation, with high throughput and low running costs (Topić Popović et al., 2017).

However, the identification requires a pure and intact colony, while the accuracy of the method may be impacted by culture media, incubation time of the bacterium, and sample preparation (Demirev et al., 1999; Saffert et al., 2011; Veloo et al., 2014). Therefore, the aim of this work was to investigate the sensibility and reproducibility of MALDI-TOF MS for accurate identification of *Ph. damselae* subsp. *piscicida*. This was tested by analysing clinical isolates grown on two different media, cultured at three different incubation times, and applied on the target plate by the direct sample spotting, the on-target extraction and by the full extraction method.

2. MATERIAL AND METHODS

Bacterial strains

The cultures used in this study were the isolates of *Ph. damselae* subsp. *piscicida* from various fish species. A total of 26 strains were used, originating from European sea bass (*Dicentrarchus labrax),* gilthead sea bream (*Sparus aurata*), and striped bass (*Morone chrysops x M. saxatilis*), farmed in land-based farms, in-shore and off-shore floating cages in Italy and Tunisia. All the strains were initially cultured on TSA medium supplemented with 1.5% NaCl (Oxoid Ltd, England UK). Their taxonomical position was established by morphological, physiological and biochemical tests, namely standard plate and tube tests and API 20E panels (bioMerieux, Marcy l'Etoile, France). API 20E tests resulted with the profile number 2005004 for all isolates. Before MALDI-TOF MS analyses, all isolates were cultured at 22°C on TSA and Blood Agar, BA (Certifikat doo, Croatia) enriched with 1.5% NaCl.

For the purposes of testing the method for differentiation between *Ph. damselae* subsp. *piscicida* and subsp. *damselae,* nine strains of *Ph. damselae* subsp. *damselae* isolated from gilthead sea bream farmed in land-based farms and off-shore floating cages in Italy and Albania were used. They were cultured and prepared under the same conditions as the subsp. *piscicida* strains.

Molecular identification was performed using multiplex PCR assay for *ure*C (subsp. *damselae*) and 16S rRNA genes (Osorio et al., 2000).

MALDI-TOF MS

The application of the isolates on the plate was performed by the direct sample spotting, the on-target extraction and by the full extraction method, after 24, 48, and 72 hours of incubation on each growth medium (TSA and BA). All the procedures were performed in triplicate for each

individual strain. A total of 1404 measurements were conducted on *Ph. damselae* subsp. *piscicida* and 486 measurements on *Ph. damselae* subsp. *damselae.*

Direct sample spotting: a single bacterial colony from each tested strain was smeared onto a 96-spot polished steel target plate (Bruker Daltonik, Bremen, Germany). They were allowed to air dry at room temperature (RT), after which 1 µL of MALDI matrix was added to each bacterial colony (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonik, Bremen, Germany).

On-target extraction: a single bacterial colony from each tested strain was smeared onto a 96-spot steel target plate. Subsequently 1 µL of 70 % formic acid (Kemika, Croatia) was added to each bacterial colony. After drying, 1 µL of MALDI matrix was added to each spot.

Full extraction: a loopful of a bacterial colony from each tested strain was suspended in 300 µL of LC-MS-grade water (Fisher Chemical, St. Louis, MO) and immediately vortexed. Further, 900 µL of 100 % ethanol (Kemika, Croatia) was added to the suspension, vortexed and centrifuged at 16 000 *g* for 2 minutes. The supernatant was discarded and the pellet recentrifuged. After discarding the supernatant, the pellet was dried at RT and resuspended in 20 µL of 70 % formic acid. The suspension was mixed by pipetting and 20 µL of acetonitrile was added, mixed and centrifuged at 16 000 *g* for 2 min. Before overlying with 1 µL of MALDI matrix, 1 µL of supernatant was added to each plate spot and allowed to dry.

MALDI-TOF MS was performed with a Bruker Biotyper (Bruker Daltonik, Bremen, Germany) system. The microflex LT mass spectrometer was calibrated with a bacterial test standard (Bruker Daltonik). Spectra were acquired in the positive linear mode between 2 to 20 kDa of mass range using FlexControl software in automatic mode. Bruker Biotyper 3.0 software (Bruker Daltonik) were used to analyse the spectra. Identification criteria were following: a log

score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated probable species identification, a score 1.700 to 1.999 indicated probable identification to the genus level, while a score of < 1.700 was considered unreliable. In order to minimize random effects, data obtained with replicate measurements were added to the calculation.

3. RESULTS

The impact of sample preparation on identification results

Three sample preparation protocols were tested for preparation of bacterial samples. The application of the *Ph. damselae* subsp. *piscicida* isolates on the plate was performed by the direct sample spotting, the on-target extraction and by the full extraction method. Samples prepared by the on-target extraction were overall better identified than by the other two protocols. The on-target extraction gave high log scores indicating highly probable species level identification, in both culture media, in all incubation times and in all replicates, when compared to the direct sample spotting, and to the full extraction method (Tables 1 and 2). Of 1,404 individual measurements in total, the on-target extraction in total gave 85.47 % and 87.18 % highly probable species level identifications for samples cultivated on TSA and BA, respectively. The direct spotting resulted in 100 % identification to the genus level in all cases, while the full

extraction method identified to the species level 97.86 % and 97.01 % of strains cultivated on TSA and BA respectively, all other factors comprised.

The impact of culture media on identification results

There was a difference regarding the culture media and successful acquisition of mass spectra (Figure 1). Identification of samples grown on BA outperformed identification on TSA for 0.64% for the direct sample spotting and the on-target extraction methods, as the BA mass spectra had the greater number of signals and the higher signal-to-noise ratio. However, when performing the full extraction, all time-points considered, samples grown on BA had 13.68 % of unreliable identifications and 11.11 % of no-identifications, compared to 5.13 % unreliables and 1.71 % no-identifications for samples grown on TSA. TSA medium enabled correct identification of *Ph. damselae* subsp. *piscicida* samples against the Biotyper database as highly probable species level identification in 49.57 % compared to 41.45 % for BA when performing the full sample extraction.

The impact of incubation time on identification results

The *Ph. damselae* subsp. *piscicida* strains were incubated for 24, 48, and 72 h on each growth medium. Samples incubated for 24 h gave better results over other incubation times as the number of successful identifications to the species level was higher (Tables 1 and 2), as well as the mass spectral quality sufficient for automatic acquisition. Reliable species identification (log scores of 2.300 to 3.000 and 2.000 to 2.299), considering every measurement for each species,

after 24 hours were 61.54 %, 84.61 %, and 53.85 % for samples grown on TSA and isolated by the direct sample spotting, the on-target extraction and by the full extraction method, respectively. However, when compared with identification results of samples grown for 48 and 72 h and isolated by the on-target extraction, the results did not change significantly (83.33 % and 87.18 % respectively). Reliable species identification (log scores of 2.000 to 3.000) after 24 hours were 76.92 %, 96.15 %, and 30.77 % for samples grown on BA and isolated by the direct sample spotting, the on-target extraction and by the full extraction method, respectively. Again, when identified by the on-target extraction, the results did not change significantly for 48 h (93.58 %), but declined for isolates grown for 72 h (71.79 %).

All measurements were performed in triplicate for each individual strain. The identical identification result for all three measurements was for: TSA medium and the direct sample spotting 9.40 %, the on-target extraction 20.94 %, by the full extraction method 12.82 %; for BA medium and the direct sample spotting 15.38 %, the on-target extraction 23.93 %, and by the full extraction method 3.74 %. The on-target extraction, therefore, for both media gave the most reliable results in terms of identical identification of replicates. The reliable identification with the highest score (log scores of 2.000 to 3.000) from the first measurement amounted to 100 % only for the on-target extraction from BA (24 h) (Figures 2, 3), and 92.31 % (48 h), whereas the on-target extraction from TSA amounted to 84.61 % (24 h), 76.92 % (48 h), and 84.61 % (72 h). If the first measurement was not reliable, the number of subsequent measurements needed for probable to highly probable species level identification was the least for the direct method after 24 h of incubation on both media.

The differentiation of *Ph. damselae* subsp. *piscicida* and subsp. *damselae* strains

MALDI-TOF MS successfully differentiated the strains under evaluation, although with variable identification results regarding the sample preparation, as presented in Table 3. We found that the score values of the subspecies *damselae* and *piscicida* differed also regarding the incubation time and the medium used. In all cases, the full extraction method was the method of choice for identification of subsp. *damselae*, as on the TSA medium it gave 100 % of probable species identification when incubated for 48 h, with an average score of 2.100, all strains considered. When using BA, the average score for the full extraction (48 h) was 2.030, and slightly increased to 2.067 for full extraction (72 h).

4. DISCUSSION

There are numerous challenges for identification of *Ph. damselae* subsp. *piscicida*, which particularly concern discrimination between closely related strains and the need for rapid identification in cases of disease outbreaks. Notwithstanding the existence of serological tests and molecular tools for its identification and differentiation from the subspecies *damselae* (Osorio et al. 2000), these challenges can be successfully tackled with the application of MALDI-TOF MS (Topić Popović et al, 2017). The proper identification depends on the quality of the acquired spectrum and the presence of the reference spectrum in the MALDI-TOF MS database. For that purpose we tested *Ph. damselae* subsp. *piscicida* strains grown on two different media, cultured at three different incubation times, and prepared for application on the

target plate by three protocols, in triplicates, with the intention to determine the optimal conditions for its identification. At this time, there is no published work on the influence of those parameters on the MALDI-TOF MS identification of *Ph. damselae* subsp. *piscicida.*

The direct spotting and the full extraction methods in this work were inferior to the on-target extraction at all time-points. On-target extraction gave high log scores from both culture media, and in all replicates. Although the direct spotting is an acceptable practice, in general it gives lower identification rates, and in such cases additional manual analysis is required (Anderson et al, 2012). For differentiating subspecies of *Ph. damselae*, Pérez-Sancho et al. (2016) proceeded with the protein extraction protocol with formic acid, skipping the step of the direct spotting. Indeed, in this work, subsp. *damselae* was best identified when using a full extraction method, however, as opposed to subsp. *piscicida* strains, only after a prolonged incubation (48 and 72 h), and with a slightly better overall identification score when using the TSA medium.

We expected that *Ph. damselae* subsp. *piscicida* would be identified successfully from both media used in this study. Despite being cultured on different media, bacteria should be identifiable by MALDI-TOF MS, as a set of low-molecular-weight constitutively expressed proteins form ions in the specific signature regardless of the type of medium. The housekeeping functions of such genes are always required although various media change metabolic needs of bacterium under cultivation (Valentine et al, 2005). Also, potential culture dependency of some mass signals might be excluded from the software analysis (Sauer et al, 2008). Mazzeo et al. (2006) found that representative signal pattern for *Ph. damselae* subsp. *piscicida* was always present in mass spectra regardless of the medium used, and MALDI-TOF MS correctly identified all *Ph. damselae* clinical isolates grown on blood agar in the work of Pérez-Sancho et al. (2016).

However, in this work *Ph. damselae* was slightly better identified when grown on blood agar, but only for the direct sample spotting and the on-target extraction methods. For the full sample extraction protocol, isolates grown on TSA were better isolated by 8 %. It is not clear why there are differences in identification success between TSA and BA media, but similar results were obtained by Walker et al. (2002) with *Staphylococcus aureus.* They observed that colonies from blood agar produced more spectral peaks, attributable to blood components.

In order to obtain good quality spectra, it is preferred to pick the bacterial cells in their log phase of growth, since MALDI-TOF MS recognizes mostly 16S ribosomal proteins (Veloo et al, 2014). We have thus tested the impact of the incubation time on the identification result. Also, all our isolates were grown at 22°C, as Austin and Austin (2007) recommend that the temperature of incubation for *Ph. damselae* be maintained at 22-25°C for optimum growth. Various incubation temperatures of gram-negative bacteria in the work of Ford and Burnham (2013) did not result in misidentifications, and we therefore speculate that a slight increase of the incubation temperature, if so set, would not influence the quality of the spectra obtained. In all cases, the strains were correctly identified after 24 h of incubation to a reliable species identification level. The identification rate was not enhanced by prolonging the incubation time, however in cases of the on-target extraction, it mostly maintained on the same level after 48 h, for isolates from both growth media, and even increased by 4 % after 72 h for isolates grown on TSA. Thus, MALDI-TOF MS procedure might be conducted as soon as the adequate growth is noted, or in case of *Ph. damselae* subsp. *piscicida* after 24 h of incubation. Although some authors (Balážova et al, 2014) report the issues with sample preparation after longer cultivation, due to cells firmly attached to the growth medium, and the residues of the medium thus possibly interfering the identification results, we did not have such problems with the growth of *Ph.*

damselae subsp. *piscicida*. Colonies grown at 72 h and later were convex, entire and easily detachable from the medium. It is reported that pleomorphism of the bacterium is pronounced in older cultures, and cells display Gram-variability in young 12-18 h cultures (Austin and Austin, 2007), features which did not impact the identification in this work.

The frequency of appearance or reproducibility gives each peak its significance as it is measured from replicate spectra used for the reference signature (Valentine et al, 2005). Reproducibility of the identification procedure is of great importance since strain differentiation (for example subsp. *damselae vs*. subsp. *piscicida*) is based on limited number of peaks. Thus, a decrease in the mass spectral quality could diminish the MALDI-TOF MS features which govern strain differentiation (Balážova et al, 2014).

The influence of a number of repeated measurements on overall identification success is rarely mentioned in the literature. In this work, the identical identification result for all three repetitions ranged from 9.40 % to 20.94 % (TSA, all sample preparation protocols) and from 3.74 % to 23.93 % (BA, all sample preparation protocols). Again, the on-target extraction had the most reliable results in terms of identical identification of replicates.

Our data show that MALDI-TOF MS protein fingerprinting is capable of identification of *Ph. damselae* subsp. *piscicida* from the database for this bacterium, irrespective of culture media and incubation time. However, there are nuances in those parameters as their optimization should be developed for a greater log score of identification results, and for the reduction of the data variation between laboratories. Recognizing the need for standardization of sample preparation, and for the purpose of obtaining good quality spectra and probable to highly probable species level identification of *Ph. damselae* subsp. *piscicida*, the authors suggest that MALDI-TOF MS be performed after 24 h of incubation, from fresh cultures. Also, we established that although the

on-target extraction yields the best results regarding sample preparation, the direct spotting should be performed as a first step in order to potentially minimize additional manual sample extraction. Sample preparation definitely plays a major role in overall result, and is crucial in distinguishing the subsp. *piscicida* from subsp. *damselae*. According to the result of this study and the relatively low number of identical readings in all replicates, we suggest that three measurements be conducted for every sample preparation protocol in order to obtain reproducible fingerprints allowing reliable identification. MALDI-TOF MS is already established as a confident method for identification of environmental aquatic bacteria. Upon further updating of the databases, and optimization of protocols for identification of bacteria directly from tissues, it will certainly become an indispensable tool in aquatic organisms' microbiology.

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431 Bargelloni, L. (2005). Direct identification of *Photobacterium damselae* subspecies *piscicida* by
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433 **Table 1.** Identification results regarding sample preparation of *Ph. damselae* subsp. *piscicida*
434 strains cultured on Tryptic soy agar (TSA) supplemented with 1.5% NaCl (as log score). The
435 samples were applied on the target plate by the direct sample spotting (A), the on-target
436 extraction (B) and by the full extraction method (C). Results presented are the average of three
437 measurements.
438

Strain ID	**24 h incubation**			**48 h incubation**			**72 h incubation**		
	A	B	C	A	B	C	A	B	C
177/04	2.009	2.088	2.067	1.947	2.046	2.165	2.001	2.035	2.048
235/04	2.040	2.044	2.047	1.905	1.965	2.109	2.003	2.032	1.905
299/C/04	2.048	1.999	1.953	1.951	2.084	2.038	1.999	2.062	1.888
319/04	2.020	2.072	2.045	1.931	2.017	2.051	2.035	1.994	2.006
79/05	1.993	1.989	2.073	1.866	2.051	2.086	1.948	1.896	1.758
114/05	1.974	2.042	2.040	2.024	2.047	2.071	1.959	2.045	1.947
189/A/05	2.016	2.028	2.071	1.889	2.097	2.050	1.979	2.025	1.954
189/C/05	2.054	1.956	2.063	1.944	2.041	1.967	1.993	2.071	2.065
256/05	1.994	2.019	2.030	2.008	2.066	2.021	2.035	1.955	1.823
314/05	2.029	1.978	2.011	2.026	2.110	2.112	2.006	2.068	1.952
328/05	2.003	2.124	2.093	2.039	1.993	2.029	2.051	2.028	2.120
82/06	2.008	2.058	2.037	2.085	2.088	1.923	2.006	2.093	1.787
262/08	1.850	2.030	1.933	2.005	1.948	2.116	1.914	1.996	1.351
243/10	2.078	2.086	1.938	2.019	2.028	2.018	2.004	2.011	2.037
97/14	2.017	2.032	1.814	2.003	2.054	2.065	1.973	2.067	1.599

325/C/14	2.032	2.065	1.939	2.101	2.105	1.570	2.045	2.061	2.063
352/B/14	1.925	2.092	2.079	1.738	2.052	1.686	1.948	2.054	1.911
395/14	1.836	2.058	1.912	1.864	2.070	1.807	1.995	2.044	1.851
396/14	1.882	2.046	1.850	1.957	1.995	1.910	2.045	2.031	2.072
399/14	2.016	2.040	1.931	1.863	2.085	1.643	1.983	2.045	1.960
277/A/15	2.015	2.076	1.757	1.979	2.037	2.003	1.934	2.077	1.950
278/16	1.898	1.998	1.975	1.925	2.043	2.019	1.914	2.046	2.029
305/15	1.815	2.085	1.931	1.829	2.041	1.919	1.994	2.049	1.856
335/15	2.041	2.033	1.859	1.898	2.123	1.921	1.969	2.011	2.074
340/16	2.015	2.133	1.809	1.955	2.031	1.952	2.039	2.046	2.039
342/A/16	2.043	2.153	1.918	1.915	2.062	2.071	1.951	2.034	1.998
% strains identified to genus/species with highly probable/probable level identification:									
genus	100	100	100	100	100	88.46	100	100	92,31
species	65.38	80.76	46.15	34.61	84.61	61.54	42.31	84.61	38.46

Table 2. Identification results regarding sample preparation of *Ph. damselae* subsp. *piscicida* strains cultured on Blood agar (BA) supplemented with 1.5% NaCl (as log score). The samples were applied on the target plate by the direct sample spotting (A), the on-target extraction (B) and by the full extraction method (C). Results presented are the average of three measurements.

Strain ID	**24 h incubation**			**48 h incubation**			**72 h incubation**		
	A	B	C	A	B	C	A	B	C
177/04	2.057	2.057	1.911	2.053	2.010	2.065	1.849	2.016	2.057
235/04	2.001	2.065	1.755	1.980	2.008	2.031	1.876	2.067	2.045
299/C/04	2.025	2.057	1.945	1.990	2.065	1.984	1.878	2.053	2.089
319/04	2.022	2.052	1.966	2.018	2.062	2.058	1.806	1.950	2.105
79/05	2.018	2.060	1.926	1.970	2.049	2.080	1.752	2.021	2.066
114/05	2.030	2.131	1.947	1.986	2.044	2.083	1.793	2.037	2.112
189/A/05	2.072	2.121	1.704	2.029	2.103	2.026	1.867	2.038	2.136
189/C/05	2.072	2.063	1.543	2.030	2.129	2.088	1.775	2.089	2.106
256/05	2.031	2.028	1.975	1.985	2.111	2.054	1.987	1.976	1.877
314/05	2.013	2.111	1.889	1.956	2.188	1.808	2.081	2.048	1.654
328/05	2.034	2.183	1.997	2.029	2.208	1.954	2.033	2.093	1.955
82/06	1.965	2.107	1.940	1.981	2.177	1.843	2.042	2.140	1.634
262/08	2.025	2.080	1.872	1.984	2.108	1.858	2.041	1.975	1.951
243/10	2.028	2.130	1.943	2.106	2.054	1.478	2.024	2.057	1.989
97/14	2.053	2.134	2.086	1.950	2.125	1.860	1.983	1.994	1.924
325/C/14	2.031	2.138	2.153	2.046	2.087	1.909	1.870	1.951	1.793

352/B/14	2.079	2.096	1.772	2.041	2.114	1.986	1.956	2.008	1.953
395/14	2.048	2.110	1.959	2.057	2.102	1.434	2.036	2.097	1.989
396/14	2.076	2.181	1.784	2.090	2.189	2.076	2.012	2.075	2.023
399/14	2.079	2.107	1.906	2.026	2.079	1.497	2.110	2.011	1.900
277/A/15	1.926	2.101	1.958	2.056	2.036	1.751	2.087	2.029	2.093
278/16	2.125	2.119	1.793	2.119	2.071	1.695	2.042	2.034	2.049
305/15	2.116	2.102	1.676	2.047	2.100	1.818	2.120	2.102	2.083
335/15	2.014	2.056	1.922	2.030	2.080	1.973	2.074	2.071	1.698
340/16	1.999	2.100	1.884	2.041	2.038	1.862	2.051	2.024	1.951
342/A/16	2.009	2.111	1.598	2.023	2.130	1.819	2.021	2.119	1.716
% strains identified to genus/species with highly probable/probable level identification:									
genus	100	100	92.3	100	100	96.15	100	100	96.15
species	88.46	100	7.69	65.38	100	34.61	53.84	80.77	46.15

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449 **Table 3**. Identification results regarding sample preparation of *Ph. damselae* subsp. *damselae*
450 strains cultured on Tryptic soy agar (TSA) and Blood agar (BA) supplemented with 1.5% NaCl
451 (as log score). The samples were applied on the target plate by the direct sample spotting (A), the
452 on-target extraction (B) and by the full extraction method (C). Results presented are the average
453 of three measurements.

454

TSA

Strain ID	**24 h incubation**			**48 h incubation**			**72 h incubation**		
	A	B	C	A	B	C	A	B	C
308/14	1.769	1.881	2.062	1.865	2.038	2.052	1.961	1.983	2.129
33/E/15	1.903	1.943	2.122	1.920	2.022	2.136	1.895	2.011	2.118
204/16	1.985	1.840	1.814	1.865	2.008	2.108	1.893	2.019	2.029
231/16 E	1.821	1.793	2.049	1.911	2.046	2.200	1.948	1.983	1.920
164/10	1.683	1.786	1.915	1.782	1.786	2.093	2.019	1.936	1.832
201/15	1.823	1.820	2.044	1.773	1.917	2.026	1.904	1.779	2.052
204/A/15	1.773	1.902	2.118	1.835	1.912	2.127	1.936	1.858	2.181
236/16	1.769	1.897	1.999	1.840	1.776	2.011	1.842	1.809	1.926
150/B/15	1.970	1.879	1.999	1.984	2.020	2.146	1.872	2.035	2.088

BA

Strain ID	**24 h incubation**			**48 h incubation**			**72 h incubation**		
	A	B	C	A	B	C	A	B	C

308/14	1.794	2.014	1.981	1.865	2.020	2.050	1.908	1.969	2.088
33/E/15	1.836	2.149	2.064	1.903	1.924	2.190	1.925	1.982	2.163
204/16	1.784	1.943	1.842	1.914	2.008	1.944	2.055	2.049	2.161
231/16 E	1.828	1.817	1.904	1.899	1.722	2.127	2.017	2.122	2.011
164/10	1.880	1.732	1.939	1.683	1.675	1.999	1.961	1.852	1.878
201/15	2.009	1.990	1.971	1.871	1.925	1.943	1.905	2.020	2.079
204/A/15	1.975	1.732	1.981	1.936	1.650	2.119	2.035	1.881	2.161
236/16	1.697	1.844	2.062	1.823	2.043	1.989	1.932	1.978	2.080
150/B/15	2.002	1.981	1.796	1.871	1.772	1.912	1.971	1.848	1.970

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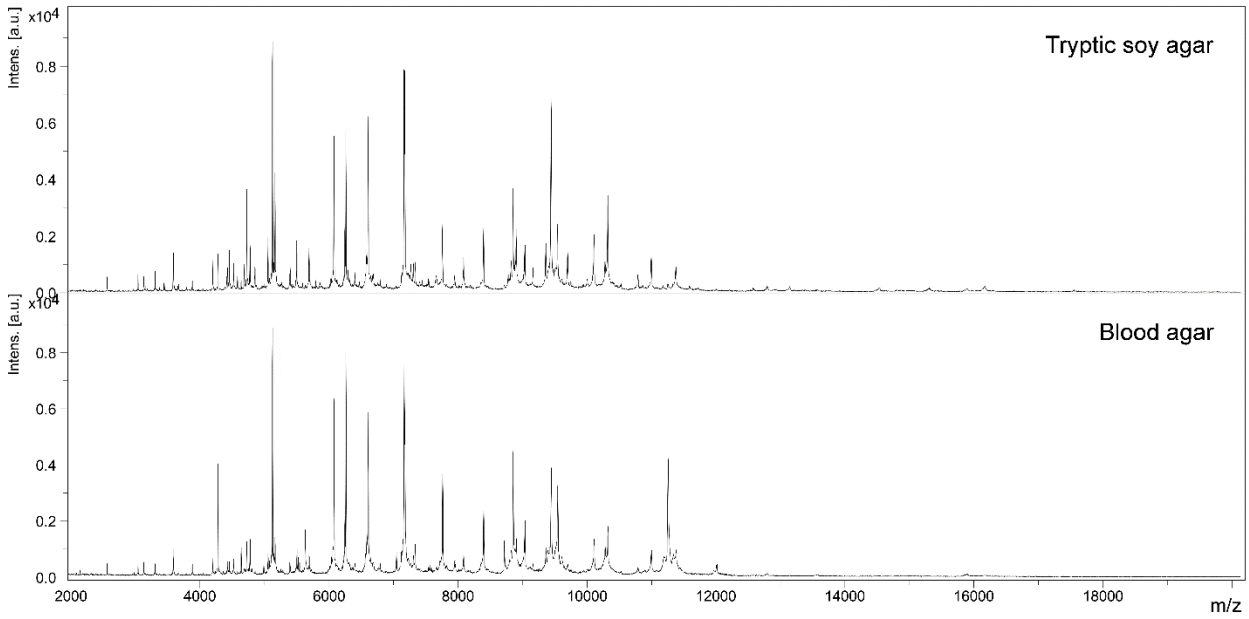
Figure 1. Representative spectra (m/z 2.000 to 14.000) for *Ph. damselae* subsp. *piscicida* for each culture medium enriched with 1.5% NaCl: tryptic soy agar, blood agar.

Figure 2. The gel view of raw spectra for 26 isolates of *Ph. damselae* subsp. *piscicida* (24h, on-target extraction, blood agar enriched with 1.5% NaCl)

Figure 3. PCA clustering results as 3D and 2D plots (scores plots and loading plots) of the spectra of tested *Ph. damselae* subsp. *piscicida* (24 h, on-target extraction, blood agar enriched with 1.5% NaCl). The isolates are clustered, demonstrating the homogeneity of the protein spectra among 26 isolates. Each dot represents the spectrum of one isolate.



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470 Fig. 1

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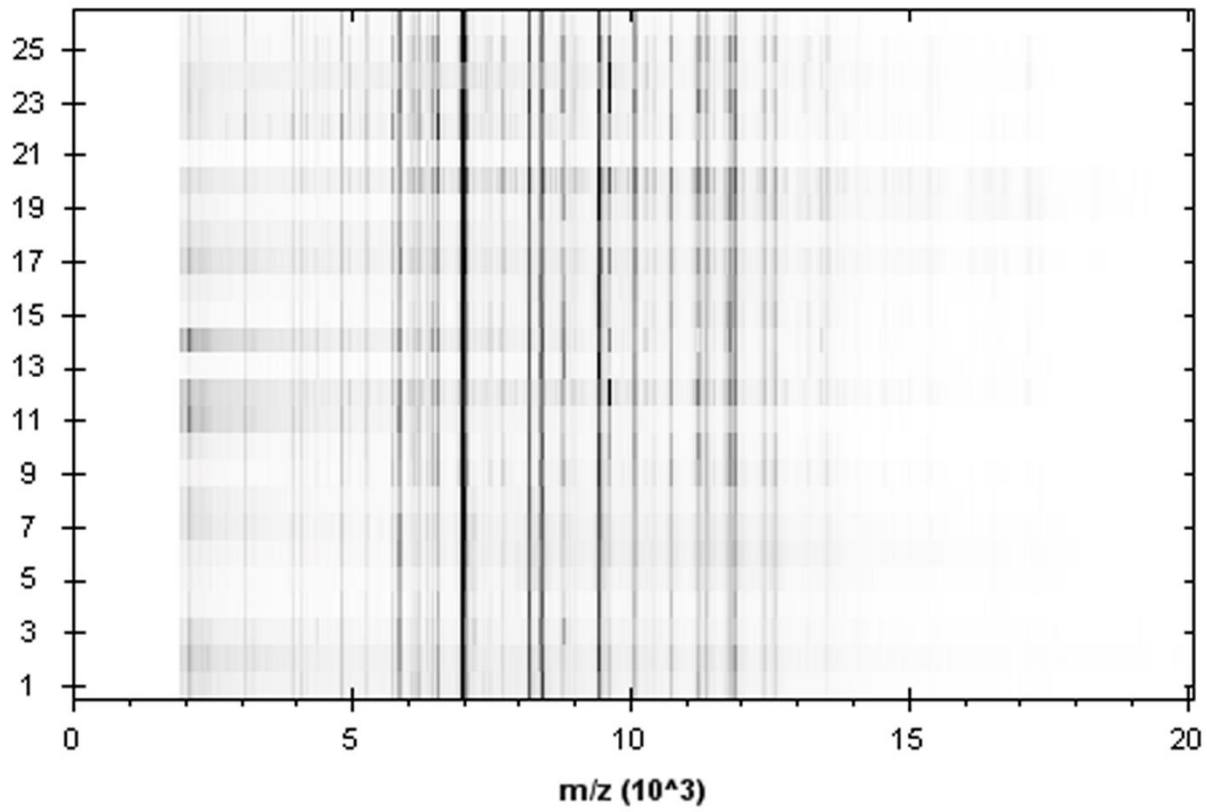

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473 Fig. 2

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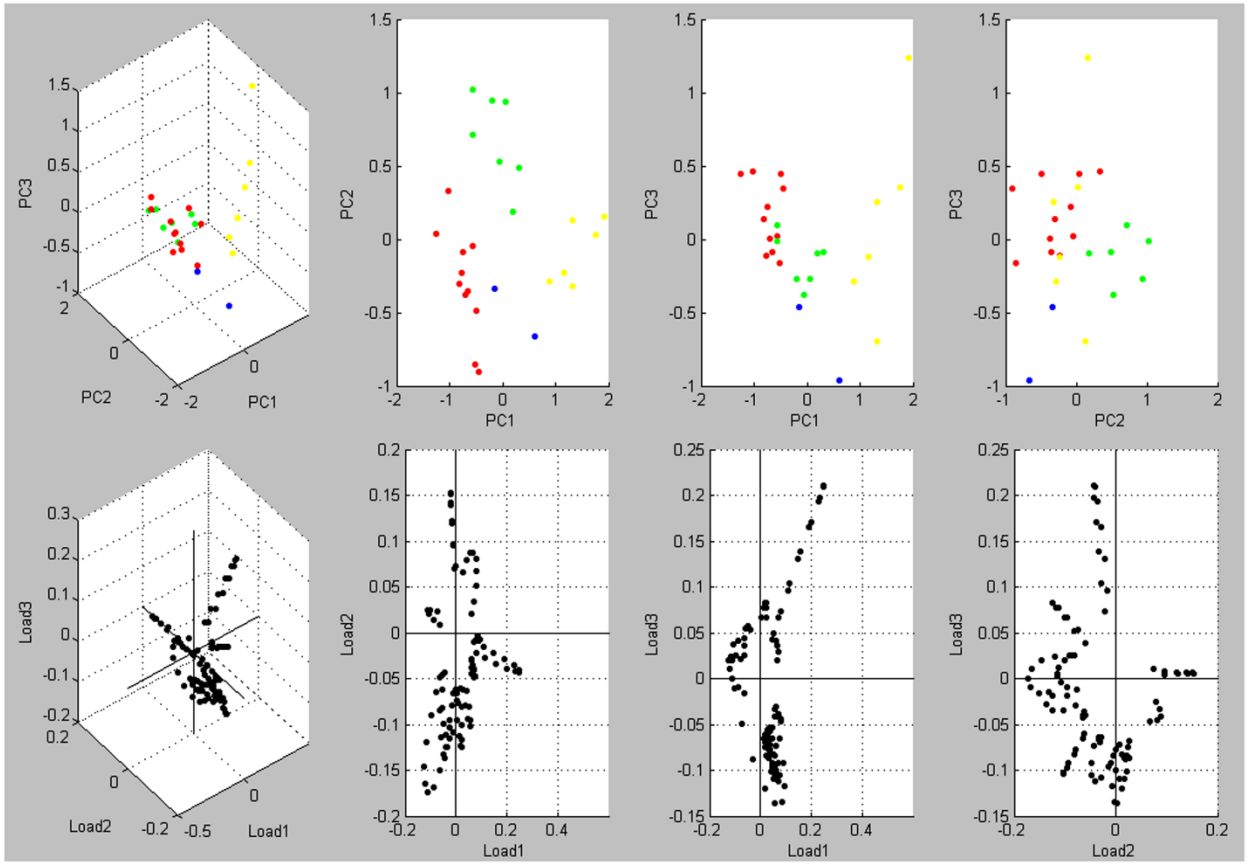

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476 Fig. 3

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