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New procedure to pre-select lactic acid bacteria able to control table-olive fermentation

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ABSTRACT. Using 35 artisanal Calabrian table-olive brines, 153 strains of lactic acid bacteria were isolated. A screening procedure was designed, involving the following steps: 1) Gram stain, cellular morphology, homolactic fermentation, acidifying activity in MRS broth; 2) growth in olive paste with NaCl 11%; 3) increase of the antioxidant activity; 4) identification of the best strains. The proposed procedure allows an evaluation of the technological aptitude of lactic acid bacteria to ferment table olives excluding, cheaply and easily, all those strains definitely unable to act as starter. The use of olive paste as a screening medium allows the resistance of the new isolates against olive phenolic compounds to be tested. The selection of strains able to increase the antioxidant power of olive paste can improve the shelf-life of table olives.

Keywords: antioxidant power; *Lactobacillus pentosus*; olive paste; screening; shelf-life; starter culture.

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Introduction

Table olives are traditional fermented products widespread in Europe, with Spain, Greece, and Italy being the main producers (Bleve et al., 2015). Greek, Spanish, and Californian methods are the main processes used to produce table olives (Tofalo et al., 2012; Catania et al., 2014; Johnson & Mitchell, 2018).

Lactic acid bacteria (LAB) and yeasts determine the table-olive fermentation process (Arroyo-López et al., 2012). LAB are responsible for a pH decrease, which inhibits the growth of pathogenic and spoilage microorganisms (Hurtado, Reguant, Bordons, & Rozès, 2012), for oleuropein hydrolysis, that removes the bitter taste of the fruit by producing hydroxytirosol (Kaltsa, Papaliaga, Papaioannou, & Kotzekidou, 2015; Ghabbour et al., 2016; Ramírez, Brenes, Castro, Romero, & Medina, 2017), and for the production of compounds that confer aroma and flavour to the final product. Yeasts play a role in flavour - since they can produce metabolites such as ethanol, glycerol, higher alcohols, and esters - and in texture formation. They help the degradation of phenolic compounds through esterase activity; moreover, they can synthesize bioactive compounds with antioxidant activity, other than synthesising vitamins, amino acids and purines that are helpful for LAB growth. Conversely, several yeasts can cause softening and gas-pocket spoilage of fruits; others can induce production of low-pigmented table olives, by adsorbing phenolic compounds responsible for their colour (Caridi, 2020).

The evolution of spontaneous fermentation in table olives is rather unforeseeable; therefore, the selection and the use of starter cultures of LAB is becoming more widespread (Aponte et al., 2012; Rodríguez-Gómez et al., 2013). Selected starter cultures of LAB must improve the microbiological control of the process, increase the lactic acid yield, and produce high-quality fermented olives (Corsetti, Perpetuini, Schirone, Tofalo, & Suzzi, 2012). Moreover, inhibition activity against undesirable microorganisms would certainly be useful (Panebianco & Caridi, 2021). Lastly, there is a growing interest in novel strain features, especially related to their effect on health.

In order to evaluate the technological aptitude of new strains of LAB to ferment table olives, different olive fermentation trials are usually carried out using the isolates.

The aim of the present study was to develop a screening procedure allowing to easily and cheaply exclude all those strains definitely unable to act as a starter culture. So, the pre-selection will indicate only a few strains to test in olive fermentation trials.

Material and methods

A total of 35 artisanal Calabrian table-olive brines of the cultivars *Carolea*, *Geracese*, *Nocellara messinese*, *Ottobratica*, *Roggianella*, and *Sinopolese* were plated at different stages of fermentation in MRS agar, thus isolating 153 strains of autochthonous LAB (Tables 1, 2 and 3).

Table 1. List of the LAB isolated from *Carolea* cultivar studied for: Gram staining, CO_2 production, cellular morphology, pH in MRS broth. Rod: bacilliform bacterium; coccus: spherical bacterium. Data highlighted in grey caused the exclusion of the strains. The strains in bold are those that passed all the tests.

Strain	*CO_2*	*Shape*	*pH*	*Strain*	*CO_2*	*Shape*	*pH*	*Strain*	*CO_2*	*Shape*	*pH*	*Strain*	*CO_2*	*Shape*	*pH*
B204	-	coccus	4.26	*B266*	-	rod	3.65	***B275***	-	rod	3.66	***B306***	-	rod	3.72
B205	-	coccus	3.69	*B267*	-	rod	5.67	*B276*	-	rod	3.66	***B348***	-	rod	3.75
B239	-	rod	3.64	*B268*	-	rod	5.86	*B277*	-	rod	3.70	*B349*	-	rod	3.75
B240	-	rod	3.63	*B269*	-	rod	3.66	*B316*	-	rod	3.71	***B350***	-	rod	3.76
B241	+	rod	3.63	*B270*	-	rod	5.76	*B317*	-	rod	3.71	*B351*	+	rod	3.74
B242	-	coccus	4.70	***B307***	-	rod	3.70	*B318*	-	rod	3.76	***B387***	-	coccus	3.75
B247	-	rod	3.77	*B308*	-	rod	3.70	*B230*	-	coccus	4.36	***B388***	-	rod	3.74
B248	-	rod	3.63	***B271***	-	rod	3.67	*B231*	+	coccus	4.43	*B389*	-	rod	3.75
B249	-	rod	3.68	*B272*	-	rod	3.66	*B232*	-	coccus	4.56	***B324***	-	rod	3.75
B250	-	rod	3.65	*B273*	-	rod	3.69	***B233***	-	coccus	3.70	*B327*	-	rod	3.75
B251	-	rod	3.67	*B310*	-	rod	3.70	***B345***	-	rod	3.74	*B393*	+	coccus	3.72
B252	-	rod	3.65	*B311*	-	rod	3.69	*B346*	-	rod	3.78	***B394***	-	rod	3.75
B253	-	rod	3.73	*B312*	+	rod	4.42	*B347*	-	rod	3.73	*B329*	-	rod	3.78
B294	-	rod	3.72	*B335*	-	rod	3.68	***B384***	-	rod	3.72	*B331*	-	rod	3.79
B295	-	coccus	3.67	*B336*	-	rod	3.72	*B385*	-	rod	3.74	***B332***	-	rod	3.74
B297	-	rod	3.66	***B330***	-	rod	3.94	*B304*	-	rod	3.78				
B298	-	rod	3.67	***B337***	-	coccus	3.70	*B305*	-	rod	3.75				

Table 2. List of the LAB isolated from *Geracese* cultivar studied for: Gram staining, CO_2 production, cellular morphology, pH in MRS broth. Rod: bacilliform bacterium; coccus: spherical bacterium. Data highlighted in grey caused the exclusion of the strains. The strains in bold are those that passed all the tests.

Strain	*CO_2*	*Shape*	*pH*	*Strain*	*CO_2*	*Shape*	*pH*
B200	-	rod	3.64	*B257*	+	rod	3.66
B201	-	rod	4.32	*B259*	-	rod	3.66
B202	-	rod	3.64	*B260*	-	rod	3.65
B216	-	coccus	3.68	*B261*	-	rod	3.65
B217	-	coccus	3.63	***B299***	-	rod	3.71
B218	-	coccus	3.63	*B300*	-	rod	3.72
B203	-	coccus	4.64	*B301*	-	coccus	4.40
B206	-	rod	3.89	*B302*	+	rod	4.52
B207	-	coccus	3.62	*B303*	-	rod	3.70
B220	-	coccus	3.63	*B390*	+	rod	3.85
B221	-	rod	3.63	***B395***	-	rod	3.70
B222	-	rod	3.62	***B358***	-	rod	3.72
B223	-	rod	3.63	*B359*	-	rod	3.72
B219	-	coccus	4.32	*B524*	-	rod	3.73
B245	-	coccus	4.85	***B525***	-	rod	3.72
B246	-	coccus	4.47	***B360***	-	rod	3.74
B291	-	coccus	4.60	*B361*	-	rod	3.71
B292	-	coccus	4.54	***B362***	-	rod	3.76
B293	-	coccus	4.62	*B391*	+	rod	3.79
B254	-	rod	3.67				
B255	-	rod	3.67				
B256	-	rod	3.65				

Table 3. List of the LAB isolated from *Nocellara* (N), *Ottobratica* (O), *Roggianella* (R), *Sinopolese* (S), and mixed cultivars (M) studied for: Gram staining, CO_2 production, cellular morphology, pH in MRS broth. Rod: bacilliform bacterium; coccus: spherical bacterium. Data highlighted in grey caused the exclusion of the strains. The strains in bold are those that passed all the tests.

	Strain	*CO_2*	*Shape*	*pH*		*Strain*	*CO_2*	*Shape*	*pH*		*Strain*	*CO_2*	*Shape*	*pH*		*Strain*	*CO_2*	*Shape*	*pH*
	B262	+	rod	5.70		*B284*	+	rod	4.44	R	*B243*	-	coccus	4.77		***B212***	-	rod	3.63
	B263	+	rod	5.80		*B285*	-	rod	4.27		*B244*	-	coccus	4.77		*B213*	-	rod	3.78
	B278	-	rod	3.78		*B286*	-	rod	4.45							*B214*	-	coccus	3.66
	B279	-	rod	3.73		***B287***	-	rod	3.70		*B208*	-	rod	3.97		*B215*	-	coccus	3.63
	B280	-	rod	3.72	O	*B288*	+	rod	4.35		*B209*	-	rod	4.37	M	*B234*	-	rod	3.61
	B281	-	rod	3.74		*B289*	+	rod	4.44		*B210*	-	rod	4.14		*B235*	-	rod	3.61
	B282	-	rod	3.72		*B290*	+	rod	4.35	S	*B211*	+	coccus	3.65		*B236*	-	rod	3.62
N	*B283*	-	rod	3.74		*B321*	+	rod	4.43		***B224***	-	coccus	3.68		***B237***	-	coccus	3.64
	B319	-	rod	3.75		*B322*	+	rod	4.40		*B225*	-	coccus	4.26		*B238*	-	coccus	4.24
	B320	-	rod	3.74		*B323*	-	coccus	4.95		*B226*	-	coccus	4.34					
	B364	-	rod	3.72							***B227***	-	rod	3.63					
	B365	-	rod	4.31							*B228*	-	rod	3.63					
	B366	-	rod	5.81							*B229*	-	rod	3.63					
	B367	-	rod	3.73															
	B368	-	rod	3.75															

A screening procedure was designed, involving the following steps.

1) Gram staining, cellular morphology, and lactic fermentation. In order to control that the strains are Gram +, study their cellular morphology, and select the strains that perform homolactic fermentation and exhibit good acidifying activity in MRS broth, each isolate was inoculated in a test tube containing 10 mL MRS broth and one Durham tube - inserted upside down - used to detect production of CO2. After incubation for 4 days at 25°C, the strains were characterized for cell morphology by optical microscope and Gram staining to exclude the duplicates among the isolates from the same sample. Obviously, this helps to reduce the number of considered strains, but it is not a guarantee to assure that they are not duplicates, since very different strains can present a similar morphology. CO2 production was evaluated based on the presence/absence of bubbles in the Durham tubes and gas-producing strains were excluded. The pH of the inoculated MRS broth test tubes was measured, excluding the strains exhibiting low acidifying activity (pH over the mean value of the totality of the samples).

2) Growth in salted olive paste. In order to evaluate the strains' ability to grow in olive paste with the addition of sodium chloride 11%, the following medium was prepared. Olive pulp of *Ottobratica* and *Carolea* cultivars and distilled water (ratio 1:2) were homogenized and sodium chloride was added to gain a final concentration of 11%. The olive paste was distributed in test tubes (14 mL tube-1), thermized at 121°C for 5' and rapidly cooled down to 4°C to reduce oxidation. Using the content of one test tube, the pH of the olive paste was measured using the supernatant after centrifugation. Then, each strain was inoculated at 2% from MRS broth in the thermized olive paste and, after mixing for 30 seconds by Vortex, incubated for 28 days at 25°C, in order to simulate fermentation at room temperature. At the end of fermentation, the fermented olive paste was centrifuged and, using the supernatant: a) the pH was measured to control the ability of LAB to grow in the olive paste; b) the total (titratable) acidity was expressed in grams of lactic acid per 100 mL brine; c) the lactic acid (D+L) content (expressed as ppm) was determined by an enzymatic reaction, using FoodLab system (CDR, Florence, Italy). The strains exhibiting low ability to grow in olive paste with the addition of sodium chloride 11% - pH over the mean value, total acidity and lactic acid content under the mean value - were excluded.

3) Ability to increase the antioxidant activity of the salted olive paste. After 28 days of fermentation, the samples of inoculated olive paste were analysed - using the supernatant after centrifugation - for the antioxidant activity by ABTS assay according to Re et al. (1999). This analysis evaluates the capacity of the sample to inhibit ABTS+ radical oxidation compared with a standard antioxidant (0-15 mM of Trolox). After preparation of the ABTS+ radical, spectrophotometric analysis was performed at 734 nm and results were

expressed as a percentage of inhibition. The strains exhibiting low ability to increase the initial antioxidant activity of the olive paste with the addition of sodium chloride 11% were excluded.

4) Identification by molecular methods. The strains exhibiting the best technological characteristics in thermized olive paste were identified by molecular methods. The steps were as follows: first amplification of the 16S rRNA gene, and then purification and sequencing. In particular, DNA from pure culture of isolates was extracted using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions and quantified by BioPhotometer D30 (Eppendorf, Milan, Italy). The identification at species level was carried out by 16S rRNA gene sequencing using fD1 (5′-CCGAATTCGTCGACAACA GAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-CCCGGGATCCAAGCTTAAGGAGGT GATCCAGCC-3′) primers (Thermo Fisher Scientific) according to Martorana, Giuffrè, Capocasale, Zappia, & Sidari (2018). Then, the PCR products were purified (GFX PCR DNA and gel band purification kit, GE Healthcare) and sequenced by Sanger method (Eurofins Genomics). The identities of the sequences were determined by Blast search tool at http://www.ncbi.nlm.nih.gov. The differentiation of closely related species *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* was achieved by multiplex PCR analysis of the *recA* gene according to Torriani, Felis, & Dellaglio (2001).

Results and discussion

Considering the literature data, an ideal starter culture of LAB for table olive production should possess a number of characteristics, such as fast growth, homo-fermentative metabolism, high acidification rate, tolerance to salts, organic acids and polyphenols, and ability to synthesize bioactive compounds with antioxidant activity (Corsetti et al., 2012).

The proposed procedure allows the cheap and easy exclusion of all the strains definitely unable to act as a starter; therefore, the first advantage offered by this procedure is the drastic reduction in the number of strains to be subsequently tested on the olives.

Figure 1 reports the four traits studied in the 153 LAB strains.

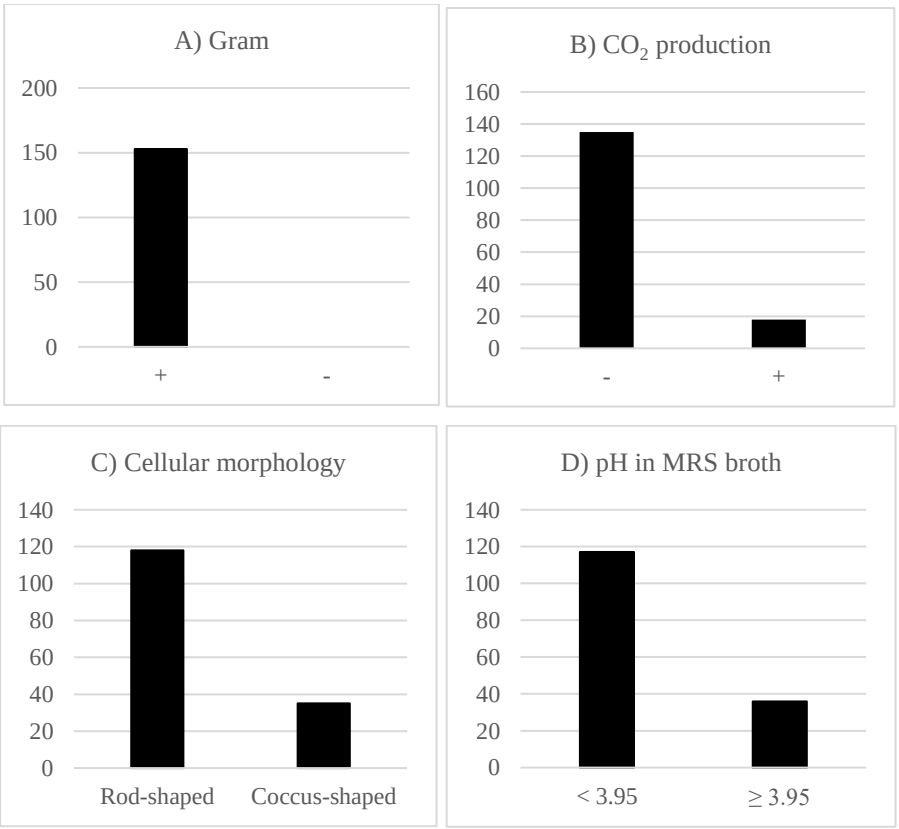

Figure 1. Frequency groups of the 153 LAB studied for: A) Gram staining to control that all the strains are Gram positives; B) CO_2 production to select strains that perform homolactic fermentation; C) Cellular morphology to exclude the duplicates among the isolates from the same sample; 4) pH in MRS broth to exclude the low acidifying strains, that produce a pH ⩾ 3.95, which is the mean value obtained in this trial from the 153 strains.

Concerning the Gram staining (Figure 1, panel A), the test confirmed that all the 153 strains are Gram positives. Concerning the CO2 production (Figure 1, panel B), 135 strains performs homolactic fermentation whereas the other 18 strains performs heterolactic fermentation. Concerning the cellular morphology (Figure 1, panel C), 118 strains are rod-shaped whereas the other 35 strains are coccus-shaped. Concerning the pH in MRS broth (Figure 1, panel D), 117 strains produced a pH < 3.95 - which is the mean value obtained in this trial from the 153 strains - whereas the other 36 strains produced a pH ⩾ 3.95. The pH ranged (as detailed in Tables 1-3) from a minimum value of 3.61 (strains B234 and B235) to a maximum value of 5.86 (strain B268). Considering these results, the 18 strains performing heterolactic fermentation and the 36 strains producing in MRS broth a pH ⩾ 3.95 were excluded. Obviously, this system is not fully comparable to table-olive production, as the MRS, even if it is an optimum enriched medium, is different to lactic acid production in a brine with olives. In addition, for each sample the strains with identical cellular morphology were excluded. However, as the use of molecular approaches has demonstrated, morphology is not always a valid parameter to separate strains, as two different strains may have the same morphology. Consequently, Tables 1-3 report, highlighted in grey, all the data that caused the exclusion of 115 strains, whereas the 38 strains that passed all the tests are in bold.

The 38 strains were tested for their ability to grow in olive paste with the addition of sodium chloride 11% (Table 4).

Table 4. List of the 38 LAB inoculated in thermized olive paste with sodium chloride 11%. After 28 days the samples were analysed towards pH, total acidity, and lactic acid content. Data highlighted in grey caused the exclusion of the strains. The strains in bold are those that passed all the tests.

N°	pH	Total acidity (% of lactic acid)	Lactic acid (ppm)	N°	pH	Total acidity (% of lactic acid)	Lactic acid (ppm)	N°	pH	Total acidity (% of lactic acid)	Lactic acid (ppm)
Control (olive paste)	4.91	0.10	163	***B271***	4.60	0.15	533	*B348*	4.64	0.10	539
B202	4.61	0.14	491	*B275*	4.61	0.11	530	*B350*	4.65	0.11	517
B205	4.80	0.11	166	***B278***	4.63	0.14	498	*B358*	4.82	0.11	151
B206	4.68	0.12	547	***B287***	4.63	0.15	502	*B360*	4.67	0.10	499
B207	4.85	0.12	187	*B295*	4.84	0.14	246	*B362*	4.76	0.11	228
B212	4.67	0.14	509	***B299***	4.65	0.15	487	*B364*	4.69	0.11	510
B218	4.75	0.11	214	*B306*	4.69	0.11	508	*B367*	4.66	0.11	536
B224	4.81	0.12	186	***B307***	4.61	0.16	576	*B384*	4.72	0.14	505
B227	4.64	0.14	425	*B324*	4.71	0.11	481	*B387*	4.70	0.11	482
B233	4.75	0.15	188	*B330*	4.72	0.16	283	*B388*	4.66	0.10	555
B237	4.74	0.12	293	*B332*	4.65	0.10	370	*B394*	4.78	0.10	181
B239	4.66	0.15	511	*B337*	4.75	0.12	256	*B395*	4.66	0.11	536
B250	4.63	0.16	532	*B345*	4.61	0.11	474	*B525*	4.73	0.12	410

The olive paste, before being inoculated with the LAB, had a pH of 4.91, a total acidity of 0.10%, and a lactic acid content of 163 ppm. After 28 days of fermentation: a) the pH ranged from a minimum value of 4.60 (strain B271) to a maximum value of 4.85 (strain B207) with a mean value of 4.70±0.07; b) the total acidity ranged from a minimum value of 0.10% (strain B348) to a maximum value of 0.16% (strains B250, B307, and B330) with a mean value of 0.12±0.02%; c) the lactic acid content ranged from a minimum value of 151 ppm (strain B358) to a maximum value of 576 ppm (strain B307) with a mean value of 412±143 ppm. Considering these results, all the strains producing a pH ⩾ 4.70, or a total acidity ⩽ 0.12% or a lactic acid content ⩽ 412 ppm were excluded. Consequently, Table 4 reports, highlighted in grey, all the data that caused the exclusion of 28 strains, whereas the 10 strains that passed all the tests are in bold. These selected strains could be thus able to complete the fermentation process for the time necessary to obtain table olives that fall within the trade standard, particularly for pH and acidity. The low acidifying strains are excluded in two steps: firstly, in a standard substrate for LAB (the MRS broth), successively in a medium that only supplies the substrates naturally present in the olives (salted olive paste). Often the strains that develop well in standard substrates do not have the same fermentation vigour when they are employed in table-olive fermentation. This usually depends on the fact that, in order to develop, they require the prior development of other microbial forms present in the first weeks of the spontaneous fermentation of table olives. Strains selected for their ability to grow and acidify the olive paste should not have any "waiting time" before beginning to develop.

The 10 strains were tested for their ability to increase antioxidant activity after 28 days of fermentation, expressed as percentage of inactivation, of the olive paste with the addition of sodium chloride 11% (Table 5).

Table 5. List of the 10 LAB studied for their ability to interact with the antioxidant power of the olives by ABTS inactivation assay. The 3 strains in bold were able to increase this parameter more and, consequently, they were identified by molecular methods. Data highlighted in grey caused the exclusion of the strains. The strains in bold are those that passed the test.

N°	ABTS (% of inactivation)
Control (olive paste)	63.03
B202	62.70
B212	59.92
B227	51.41
B239	67.68
B250	68.11
B271	63.54
B278	64.22
B287	62.65
B299	64.49
B307	73.41

The olive paste, before inoculation with the LAB, had a percentage of inactivation of 63.03%. The percentage of inactivation, after 28 days of fermentation, ranged from a minimum value of 51.41% (strain B227) to a maximum value of 73.41% (strain B307) with a mean value of 63.81%, very close to that of the olive paste. Since the strains exhibiting low ability to increase the initial antioxidant activity of the olive paste must be excluded, all the strains producing a percentage of inactivation ⩽ 67.03% - that is, less than 4% more than the control - were excluded. Consequently, Table 5 reports, highlighted in grey, all the data that caused the exclusion of 7 strains, whereas the 3 strains that passed the test are in bold.

According to the sequencing and the multiplex PCR, the three strains B239, B250, and B307 were identified as *L. pentosus*.

Conclusion

The advantage of the proposed two-step procedure is the rapid reduction in the number of strains under screening to be subsequently tested on the olives, and the exclusion of strains definitely unable to act as a starter for low acidifying ability, production of gas, low antioxidant activity.

The use of olive paste as a screening medium allows simultaneous testing for the resistance of the LAB against the phenolic compounds present, preventing stuck fermentation.

The addition of a significant amount of sodium chloride (11%) to the growth substrate constitutes another important barrier; obviously, in relation to the specific needs of pre-selection, this concentration could also be lower.

Last but not least, the choice to select strains that increase the antioxidant power of the olive paste could increase the shelf-life of the table olives produced, with obvious commercial and nutritional advantages.

Obviously, this protocol can be optimized based on the specific needs of each researcher; like any protocol, it may be adapted to specific territorial needs.

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