RESOLUTION OENO 15/2003

LACTIC BACTERIA

THE GENERAL ASSEMBLY,

CONSIDERING Article 5, paragraph 4 of the International Convention of the Unification of Methods of Analysis and Appraisal of Wines of 13 October 1954,

UPON PROPOSAL of the Sub-commission of Methods of Analysis and Appraisal of Wines,

DECIDES to replace the existing monograph by the following monograph in the International Oenological Codex:

LACTIC BACTERIA

1. OBJECT, ORIGIN AND FIELD OF APPLICATION

Lactic bacteria are used in oenology to perform malolactic fermentation. The lactic bacteria must belong to the Oenococcus (*Leuconostoc), Lactobacillus and Pediococcus* genus and must be isolated from grapes, musts, wine or cultures originating from the crossing of these same bacteria (original mother culture) which must be stored in genetically stable conditions.

Obtaining and using genetically modified bacteria (GMO's) require advance authorisation from a competent authority.

Lactic bacteria used in oenology must transform the malic acid in must and wine into lactic acid and carbon dioxide. This must produce biogenous aminos in the smallest possible quantities, and must neither produce an off taste nor produce substances harmful to health.

2. LABELLING

The following information must be indicated on the label:

- The genus and species name in addition to the reference of the strain(s) attributed by an official body of monitoring micro-organisms or by international institutions, the breeder, the origin, the strain breeder and possibly the originator that isolated it.

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- Operating instructions or the reactivation method and possible additives recommended by the manufacturer
- The number of viable cells per gram of preparation that is guaranteed by the manufacturer, the loss of viability per month of storage under defined conditions for temperature, humidity and aeration, the batch number, in addition to the expiration date and storage conditions.
- Where relevant, the indication that lactic bacteria were obtained by genetic modifications and their modified character.

3. CHARACTERISTICS

Lactic bacteria are used in liquid, frozen or powder form obtained by lyophilisation or drying, in pure culture or in association with pure cultures.

4. TEST TRIALS

4.1. Humidity

Measured by the weight loss of 5 g of the product, dried at 105°C until constant weight (about 3 hours).

Maximum content should not exceed 8%.

4.2. Heavy metals

Proceed with the dosage according to the method in chapter II of the International Oenological Codex.

Content should be less than 10 mg/kg of dry matter, expressed in lead.

4.3. Lead

Proceed with the determination according to the method in chapter II of the International Oenological Codex.

Content should be less than 5 mg/kg of dry matter.

4.4. Mercury

Proceed with the determination according to the method in chapter II of the International Oenological Codex.

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Content should be less than 1 mg/kg of dry matter.

4.5. Arsenic

Proceed with the determination according to the method in chapter II of the International Oenological Codex.

Content should be less than 3 mg/kg of dry matter.

4.6. Cadmium

Proceed with the determination according to the method in chapter II of the International Oenological Codex.

Content should be less than 1 mg/kg of dry matter.

4.7. Mycotoxins[1]

4.8. Viable lactic bacteria

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

The number should be more or equal to 10^8 CFU/g or 10^7 CFU/ml.

4.9. Content of viable cells of lactic bacteria of a different species of an indicated strain[2]

4.10. Mould

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

The number should be less than 10^3 CFU/g of powder.

4.11. Contaminant acetic bacteria

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

The number should be less than 10^3 CFU/g of powder or 10^3 CFU/ml. The sum of *Acetobacter* + *Gluconobacter* should be less than 10^3 CFU/g of powder or 10^3 CFU/ml millilitre.

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4.12. Yeasts

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

The number of viable cells of total contaminant yeasts (for example *Shizosaccharomyces* or *Brettanomyces*) must be less than 10^3 CFU/g of powder or 10^3 CFU/ml.

4.13. Salmonella

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

Absence should be checked on a 25 g sample.

4.14. Pseudomonas aeruginosa[3]

4.15. Escherichia coli

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

Absence should be checked on 1 g sample.

4.16. Staphylococci

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

Absence should be checked on 1 g sample.

4.17. Coliforms

Proceed with counting according to the method in chapter II of the International Oenological Codex (method in the annex of this resolution).

The number of coliforms should be less than 10 CFU/g.

5. ADDITIVES

They must be in conformity with regulations in force.

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6. STORAGE CONDITIONS

Storage should not be in open packaging and/or at temperatures above 10°C.

a. Storage conditions differ according to preparation and packaging methods.

Always refer to manufacturer's recommendations.

MICROBIOLOGICAL ANALYSIS METHODS

(to appear in chapter II of the International Oenological Codex).

1. Pemliminary rehydration of bacteria

- Under sterile conditions weigh 1 g of lactic bacteria,
- Under sterile conditions add 100 ml of sterile water at room temperature (25°C),
- Homogenise using a magnetic plate for 5 minutes,
- Leave for 20 minutes at room temperature (20°C),
- Homogenise for 5 minutes at room temperature (20°C),
- Take 10 ml under sterile conditions and proceed with micro-biological controls.

2. Determination of the number of viable lactic bacteria.

2.1. MTB/s agar medium

Composition:
Glucose: 15 g
Peptone: 8 g
Yeast extract : 5 g
Casein hydrolysate: 1 g
Tomato juice: 20 ml
Na acetate: 3 g

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NH_4 citrate : 2 g

Malic acid: 6 g

Mg sulphate: 0.2 g

Mn sulphate: 0.035 g

Tween80 : 1 ml

Minimal Eagl TC vitamin: 10 ml

after sterilisation

adjust pH 5.0 and add

Agar: 2%

Water q.s.f. : 1000 ml

potassium sorbate (400 mg/l in liquid medium) or add directly to the Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v

Sterilisation at 120°C for 20 min.

Incubate in anaerobic conditions to contrast moulds at 25°C for 8 to 10 days.

2.2. Milieu Man, Rogosa and Sharpe (MRS)

The bacteria are cultivated in a MRS liquid medium (Man, Rogosa, Sharpe 1960) and the composition is as follows:

Agar agar: 15 g

Bacto-peptone: 10 g

Meat extract: 10 g

Yeast extract: 5 g

Sodium acetate: 5 g

K_2 HPO_4: 2 g

Trisodium citrate: 2 g

$MgSO_4$ at 100 mg/l: 2.5 ml

$MnSO_4$ at 20 mg/l: 2 ml

Tween 80: 1 ml

DL malic acid : 5 g

Concentrated tomato juice*: 20 ml

Glucose: 20 g

Adjust HCl or NaOH s.p.: pH 4.8

Distilled water q.s.f.: 1000 ml

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Autoclave at 120°C for 20 min.

Potassium sorbate (400 mg/l in liquid medium) or add directly to the Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v

Incubate at 25°C for 8 to 10 days under anaerobic conditions.

*tomato juice is used to increase lactic bacterial growth.

preparation: take tomato juice in a can containing at least 7 g/l of NaCl (maxi 9 g/l)

Centrifuge at 4000 g for 20 min,

Collect clear juices and filter on paper filter,

Place in an autoclave at 120°C for 20 min.

3. Mould

Czapeck-Dox/s agar environment

Composition:

Agar agar: 15 g

Saccharose : 30 g

$NaNO_3$: 3 g

K_2HPO_3: 1 g

$MgSO_4$: 0.5 g

KCl: 0.5 g

$FeSO_4$: 0.01 g

Potassium sorbate: 0.4 g

Water: q.s.f. 1000 ml

Adjust: pH 3.5

Sterilisation at 120°C for 20 min.

Add directly to the Petri dish 0.1 ml of the penicillin solution at 0.25% in pure alcohol.

Aerobic incubation at 25 °C for 10 days

4. Contaminant acetic bacteria

4.1. Contaminant acetic bacteria

Act/s agar environment

Composition:

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Bacteriological agar agar: 20 g
Yeast extract: 5 g
Caseine amino acids: 5 g
Glucose: 10 g
Adjust to pH 4.5
Water q.s.f. 1000 ml
Sterilisation at 120°C for 20 min.
Incubate under aerobic conditions at 25°C for 7 days.
Potassium sorbate (400 mg/l in liquid medium) or
Add directly to Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v

4.2. For research of *Acetobacter*

Acb/s agar environment
Composition
Yeast extract : 30 g
Alcohol 95% per volume: 20 ml
Bromocresol green (sol. 2.2%): 1 ml
Agar: 2%
Water q.s.f. 1000 ml
Sterilisation at 120°C for 20 min.
Add directly to Petri dish 0.1 ml of penicillin solution at 0.25% in pure alcohol.
Add directly to Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v.
Incubate under aerobic conditions at 25 °C for 7 days.

4.3. Search for Gluconobacter

Gcb/s agar medium
Composition
yeast autolysate: 10 g
glucose: 3 g
$CaCO_3$: 3 g
Agar: 2%
Water: q.s.f. 1000 ml
Sterilisation at 120 °C for 20 min.

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Add directly to Petri dish 0.1 ml of penicillin solution at 0.25% in pure alcohol.
Add directly to Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v.
($CaCO_3$ facilitates the recognition of *Gluconobacter* colonies which dissolve and produce a lighter circular zone around the colony.)
Aerobic incubation at 25°C for 7 days.

5. Counting of yeasts

5.1. YM agar environment (MALT WICKERHAM)

Composition:
Bacteriological agar agar: 15 g
Yeast extract: 3 g
Malt extract :3 g
Peptone: 5 g
Glucose: 10 g
Water q.s.f. 1000 ml
Before use, the environment is autoclaved at 120°C for 20 minutes.
After inoculation, the dishes are incubated at 25°C in anaerobiosis for 48 to 72 hours.
Count the number of CFU and refer to weight of dry matter.

5.2. YMS agar medium

Composition:
Agar: 20 g
Glucose: 20 g
Yeast extract: 5 g
Malt extract: 3 g
Peptone: 2 g
Malic acid: 4 g
Grape juice: 100 ml
Vitamin complex*: 1%
Water q.s.f. 1000 ml
Before use, the environment is autoclaved at 120°C for 20 minutes.
After inoculation, the dishes are incubated at 25°C in anaerobiosis for 48 to 72 hours.

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* Vitamin complex (inositol 25 mg, biotin 0.02 mg, Ca pantothenate 4 mg, folic acid 0.002 mg, nicotinamide 4 mg, paraminobenzoic acid 2 mg, B6 hydrochloride 4 mg, riboflavin 2 mg, thiamine 10 mg, water s.p. 1000)

5.3. OGA medium

Composition:

Extract of autolytic yeast: 5 g

Glucose: 20 g

Bacteriological agar agar: 15 g

Water q.s.f. 1000 ml

Autoclave at 120°C for 20 min.

After inoculation, the dishes are incubated at 25°C in aerobiosis for 48 to 72 hours.

Count the number of CFU and refer to the weight of dry matter.

6. Count of Salmonella

6.1. Principle

The sample undergoes a pre-enrichment phase in buffered peptoned water for 16 to 20 hours at 37°C. Then the aliquot part of this mixture is inoculated for culture. This contains a specific medium and 2 special tubes (made up of 2 parts) and is incubated 24 hours at 41°C. S*almonella* migrate from the bottom (selective medium) to the top part of the tube (indicator medium). The presence of *Salmonella* is indicated by a change in colour of this solution.

6.2. Apparatus and analytical conditions

Preparation for culture is carried out in the sterile zone ensured by a Bunsen burner. The soiled material is submitted for destruction by autoclave for 1 hour at 120°C or by total immersion in a bleaching agent for at least 18 hours. (See cleaning procedure).

Sterile glass test tube 125 ml

sterile stomacher bag

Closing barrette stomacher

Sterile glass tubes 16x160 mm

Cottoned glass test tubes 20x220 mm

2 ml graduated by 0.1 ml sterile plastic pipettes

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10 ml graduated by 0.1 ml sterile plastic pipettes
Tube shaker
Method for culture to be rehydrated.
2 ml sterile needle with plastic sterile syringe.
Tweezer forceps
Wrench for unscrewing tubes A and B for culture method
Clean glass slide
Sterile cottoned Pasteur pipettes
Wire hoop
Oven at 41°C ± 1°C
Oven at 37°C ± 1°C
Bunsen burner

6.3. Reagents

Sterile peptoned water (SPW)
Sterile distilled water (SDW)
Sterile 500 ml sealed flask filled with 125 ml of SPW
Sterile 500 ml sealed flask filled with 225 ml of SPW
Special environment for *Salmonella*: SRTEM
Novobiocin disk (1.8 mg de novobiocin)
Hektoën agar agar (see DOMIC-08)
API 20E gallery
Agar agar tubes TSAYE inclined
Sterile NaCl solution at 8.5 g/l
Anti-*Salmonella* serum

6.4. Procedure

6.4.1. Preparation of reference suspension

This differs according to the nature of products and dilution rate.
Add a test portion of 25 grams or millilitres of the product in a stomacher bag to a nine fold greater amount of peptoned water.
Close the bag by heat welding or using a barrette.
Grind in the stomacher for 1 minute.

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6.4.1.1. Pre-enrichment phase in a non selective liquid medium:

Incubate the reference suspension for 16 to 20 hours at 37°C ± 1°C.

6.4.1.2. Enrichment in selective liquid mediums

Preparation of culture methods

- Unscrew the lid of the culture container;
- Add SDW up to line 1 as marked on the container.

Note: The base of tubes A and B must be located under water level.

- Adapt the needle to the syringe and check that the syringe plunger is pushed in (absence of air);
- Vertically introduce the needle of the syringe into the rubber disc in the centre of the stopper in tube A (blue stopper). Check that the needle is visible under the stopper;
- Carefully pull on the syringe body up until the liquid reaches line 3 on the container.

Note: Do not draw up liquid into the syringe.
This operation should take approximately 5 seconds.

- Repeat this operation with tube B (red stopper);
- Close the stopper from the culture container tightly;
- Press the side of the recipient on a tube shaker and maintain at least 5 seconds.

Note: the liquid in tubes A and B must be shaken vigorously.

- Allow the culture to stand at least 5 minutes;
- Unscrew the culture container's stopper and pour in the SRTEM medium until the level reaches line 2 as marked on the container;
- Add a novobiocin disc using tweezer forceps;
- Remove the stoppers from tubes A (blue) and B (red) using a wrench, then

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dispose of the stoppers.

Note: avoid touching the tubes and the inside walls of the container.

- Inoculation of culture container
- Homogenise the pre-enriched culture;
- Identify the culture container. Write down the analysis number on the lid.
- Unscrew the lid.
- Using a 2 ml pipette introduce 1 ml of pre-enriched culture in the culture container.
- Screw the lid onto the culture container.
- Write down the time and date of incubation.
- Incubate 24 hours ±30 min at 41°C ± 1°C in a strictly vertical position.

6.4.2. Reading and interpretation

This is carried out by observing the top part of A tubes and B through the container walls.

The possible presence of *Salmonella* is characterised by modifications in indicator medium colour located in one or both of the top parts of the tubes:

REACTION	TUBE A	tube B
Positive:	All degrees of black colouring	All degrees of red or black colouring
Negative:	Absence of black colouring	Absence of red or black colouring

Tubes showing a positive reaction are subjected to selective agar isolation.

- Dry boxes of Hektoën agar in an incubator at 46°± 1°C until the drops on the surface of the medium disappear completely (lid removed and agar surface facing down)
- Take a wire hoop from the positive middle indicator and inoculate it into 5 ml of

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SPW, in a 16x160 mm sterile glass tube in order to dilute the culture.

- Proceed as such with each positive tube.
- Identify the dish and write down on the lid the analysis number and the letter of the tube being confirmed.
- Homogenise the culture dilution and take a wire hoop.
- Isolate the Hektoën agar on the surface to enable the development of isolated colonies.
- Incubate 24 hours at 37°□ 1°C.
- Select at least 2 isolated colonies considered to be typical.

6.4.3. Confirmation

6.4.3.1. Biochemical tests

- Identify the different colonies by using specific miniaturised galleries
- (API 20^{E} gallery) by referring to the recommendations of the manufacturer.
- Incubate 24 hours at 37°C□ 1°C.
- At the same time inoculate: an agar to confirm the purity of the strain.
- 1 inclined TSAYE agar for serological typing.
- Incubate 24 hours at 37°C□ 1°C.
- Read the API20E gallery following the manufacturer's indications.
- Compare the profile obtained to the standard profiles given by the manufacturer.
- Store TSAYE agar in the refrigerator until utilisation.

6.4.3.2. Serological tests:

Tests are conducted when the strain profile corresponds to *Salmonella* following the recommendations defined by the manufacturer from cultures obtained on agar and after eliminating self-agglutinating strains.

Elimination of self- agglutinating strains:

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- Place a drop of saline solution at 8.5 g/l on a perfectly clean glass slide.
- Disperse a little bit of the culture taken from the nutritive agar to obtain a homogeneous and cloudy solution using a Pasteur pipette.
- Oscillate the slide for 30 to 60 seconds.
- On a black background using a magnifying glass: if observation reveals more or less distinct clusters, the strain is considered as being self-agglutinating and should not be subjected to serological typing.

6.5. Results

According to the results based on the interpretation of biochemical and serological testing, the results are expressed as follows:

- Presence of *Salmonella* in m grams or ml of product.
- Absence of *Salmonella* in m grams or ml of product.

Diagram of procedures

PREPARATION		weigh m grams or millilitres of sample
of		add v times m grams or millilitres of SPW
reference suspension	*grinding*	1 minute in a stomacher
		↓
↓PrE-enrichMENT	*incubation*	16 to 20 hours at 37°C □ 1°C
↓enrichment		1ml of pre-enrichment solution
		Ready to use culture recipient

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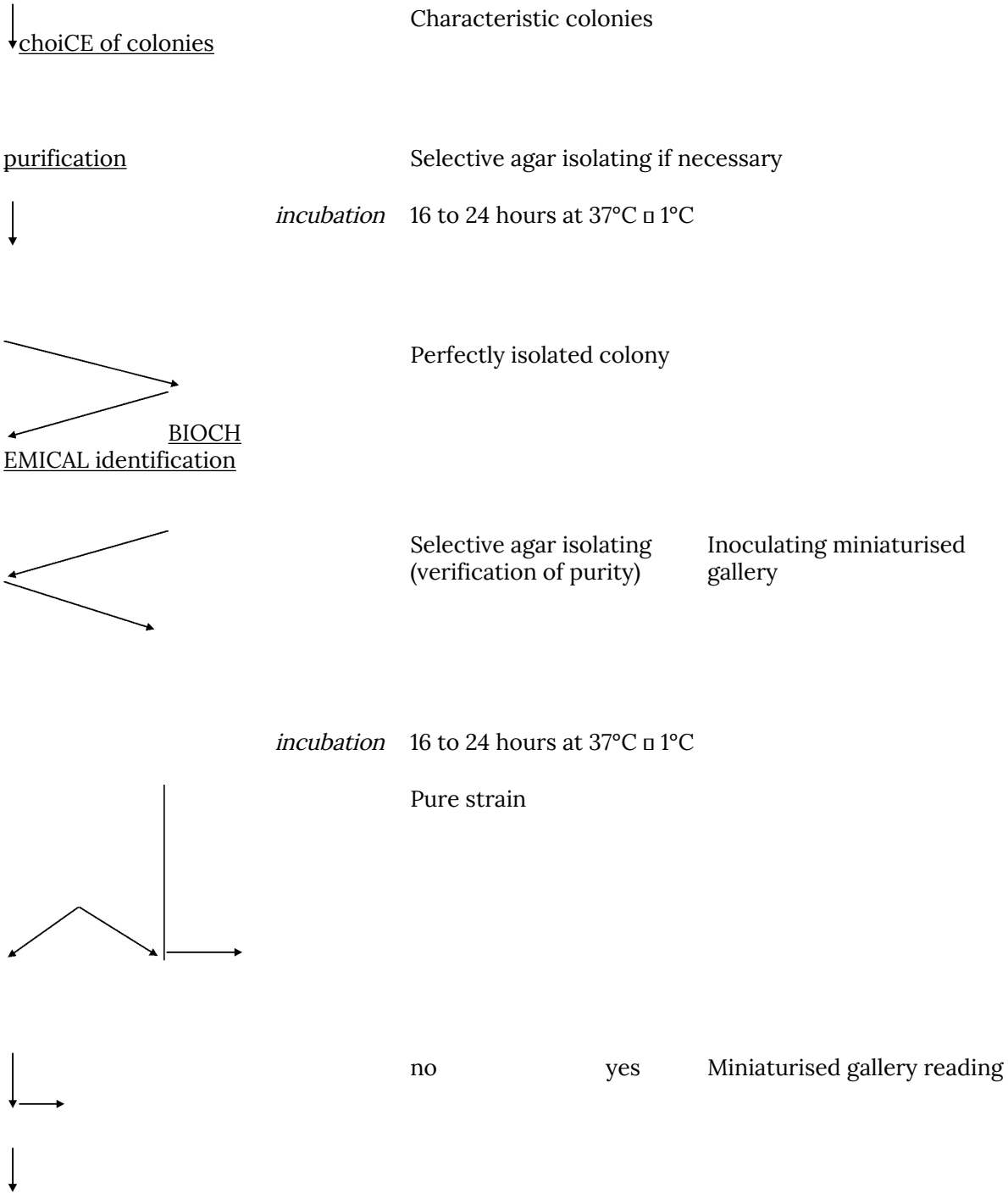
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↓	*incubation*	24 hours ▫ 30 minutes at 41°C ▫ 1°C			
READING		Top section of the tube			
↘ ↙		**A**		**B**	
INTERPRETATION	*coloration*	other	black	red or black	other
↓		▫	▫	▫	▫
↓↓↓					
INTERPRETATION		absence	**presence**	**presence**	absence
↓↓					
↓ confirmation			1 wire hoop in 5 ml of SPW		
↓					
		Isolating in striations on selective agar			
	incubation		16 to 24 hours at 37°C ▫ 1°C		
↓↓					
	choice of colonies	At least 2 characteristic colonies from each dish			
↓					
		See confirmation test diagram			

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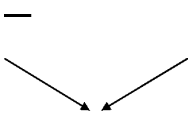
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Test confirmation diagram



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purification — *Salmonella* profile

Serological testing — yes — other

serological identification — *subculture* — On inclined TSAYE agar



- 1 tube for serology

serology — absence of auto-agglutination and antigen agglutination O:

Salmonella

Diagram of biochemical and serological interpretations

Biochemical reactions	Self-agglutination	Serological reactions	Interpretation
typical	no	"O" positive antigen	*Salmonella*
typical	no	Negative reactions	Sent to an authorised centre
typical	yes	Not carried out	for determination of the serological type

7. Count of *Escherichia coli* by the counting of colonies obtained at 44 °C

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7.1. Principle

Inoculating in Rapid E. *coli* agar in depth is carried out in a Petri dish for each of the dilutions chosen. Following a 24 hour incubation at 44°C, all characteristic colonies which appear are counted.

7.2. Apparatus and analytical conditions

Cultures are carried out in a sterility zone ensured by the usage of a Bunsen burner.
Plastic sterile Petri dishes with a diameter of 90 millimetres
Sterile 16x160 cottoned glass test tubes
Tube holder
2 ml plastic sterile pipettes with 0.1 ml graduations
Water bath at 100°C ± 2°C
Water bath at 47°C ± 2°C
Tube shaker
Oven at 44°C ± 1°C
Bunsen burner
Colony counter

7.3. Reagents

Sterile diluent for decimal dilutions: tryptone salt (TS)
16x160 pre-filled sterile tubes with 9ml of sterile TS
Rapid'E.*coli* cooling agar (R.E.C)

7.4. Procedure

7.4.1. Agar agar medium

- Melt R.EC agar in a boiling water bath. Avoid overheating.
- Never use a culture medium above 50°C.
- For immediate usage, maintain the agar in the water bath at 47°C ± 2°C.
- Do not supercool over 8 hours.
- For a deferred usage maintain the supercooling agar in an oven at 55°C ±1 °C.
- The melted culture medium not used within 8 hours will not re-solidify for another

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usage.

7.4.2. Culture

- Homogenise each dilution before inoculation in Petri dishes and before carrying out decimal dilutions.
- Transfer 1 ml from the reference solution and/or the retained decimal dilutions in the respective Petri dishes. Change the pipette after each dilution.
- Introduce at least 20 minutes after inoculum, 15 to 20 ml of R.EC maintained in the water bath at 47°C ± 2°C.
- Slowly homogenise by shaking.
- Let solidify on the bench (lid up).
- Pour 4 to 5 ml of R.EC maintained at 47°C ± 2°C.
- Let solidify on the bench (lid up).
- Return the dishes and incubate in an oven 24 hours ± 2 hours at 44°C ± 1°C.

7.4.3. Count

Dishes containing between 15 and 150 characteristic colonies of two successive solutions are retained for counting.

If the dish inoculated with 1 ml of the first dilution contains characteristic colonies and fewer than 15, it will be retained for counting.

Characteristic colonies are counted using a counter or are counted manually after 24 hours □ 2 hours of incubation.

7.5. Results

7.5.1. General case

The dishes contain between 15 and 150 characteristic colonies for two successive dilutions.

7.5.1.1. Method of calculation

The two dishes retained have between 15 and 150 characteristic colonies. The number N of counted micro-organisms at 44.5°C per millilitre (ml) or by gram (g) of product is

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obtained by calculating the weighted mean on 2 dishes retained.

$$N = \frac{\sum c}{1{,}1d}$$

□c : sum of characteristic colonies counted on the 2 dishes retained
d : rate of dilution corresponding to the first dilution

7.5.1.2. Expression of results

- Round off the number N to 2 significant digits
- Express to the tenth power , ex.: 1.6 10^3 / g or ml

7.5.2. Estimation of small numbers

If the dish inoculated with 1 ml of the 1st retained solution for analysis includes less than 15 characteristic colonies, express the result as follows:

$$N = c\frac{1}{d}$$

c : sum of characteristic colonies counted
d : rate of dilution
If the dish inoculated with 1 ml of the 1st retained solution for analysis does not contain any colonies, express the result as follows:
$N = < 1\frac{1}{d}$ micro-organism per g or ml
d : rate of dilution

8. Count of Staphylococci with a positive coagulase by the counting and confirmation of colonies obtained at 37°C

8.1. Principle

Decimal dilutions and inoculations on the surface of 1 Baird Parker agar drawn previously in a Petri dish with each of the dilutions retained, are carried out

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simultaneously from the sample (liquid product) or from the reference solution (other products).
After an incubation of 48 hours at 37°C the characteristic and/or non characteristic colonies that appear are counted and then confirmed by the coagulase test.

8.2. Apparatus and analytical conditions

Cultures are carried out in a sterility zone ensured by the usage of a Bunsen burner.

- Sterile glass 16x160 cottoned test tubes
- Sterile plastic precipiting tubes with plastic stoppers
- Tube holder
- 2 ml plastic sterile pipettes with 0.1 ml graduations
- Sterile plastic spreader
- Sterile Pasteur pipettes
- Tube shaker
- Incubator at 37°C ± 1°C
- Bunsen burner
- Colony counter

8.2.1. Reagents

- Sterile diluent for tryptone salt (TS) decimal dilutions
- 16x160 sterile tubes pre-filled with 9ml of sterile TS.
- Baird Parker agar pre-poured in a Petri dish.
- Tubes pre-filled with 5ml brain heart bouillon (sterile).
- Plasma of lyophilised rabbit to be rehydrated at the time of use.

8.2.2. Procedure

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8.2.2.1. Culture

- Dry the agar plates in an incubator at 46°C 1°C until the droplets on the surface of the environment have completely disappeared (cover is removed and the agar surface is turned downwards).
- Homogenise each dilution prior to inoculation of the agar plate surface before carrying out decimal dilutions.
- Place 0.1 ml of reference solution and/or the retained decimal dilutions on the agar surface. Change the pipette after each dilution.
- Carefully spread the inoculum as quickly as possible using a spreader without touching the edges of the dish.
- Leave the dishes with the lids on for 15 minutes at room temperature.
- Incubate 48 hours ± 2 hours at 37°C ± 1°C

8.2.2.2. Counting

Dishes containing less than 150 characteristic and/or non-characteristic colonies on two successive dilutions are retained, but one of them must include at least 15 colonies. The characteristic and/or non-characteristic colonies are counted either manually or by using a counter.

Characteristic colonies after 48 hours ± 2 hours of incubation:

- Black or grey, shiny or convex with at least a 1 mm diameter and a maximum of 2.5 mm in diameter outlined with lightening and precipitation halos.

Non-characteristic colonies after 48 hours ± 2 hours of incubation:

- Black and shiny with or without a white edge with lightening and precipitation halos that are absent or barely visible.
- Grey without light zones.

8.2.2.3. Confirmation

Take 3 characteristic colonies or 3 colonies of each type (characteristic or non-

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characteristic) and submit them to the coagulase test.
Coagulase test:

a. Bouillon culture:

- Take part of the selected colony using a Pasteur pipette sterilised with the Bunsen burner flame and inoculate into a brain heart bouillon.
- Repeat this manipulation for the other selected colonies.
- Identify the tubes by sample number and its dilution with a blue marker for characteristic colonies and a green marker for non-characteristic colonies.
- Incubate at 37°C ± 1°C for 20 to 24 hours □ 2H.

b. Testing for free coagulase:

- Add 0.5 ml of the culture obtained in brain heart bouillon to 0.5 ml of rehydrated rabbit plasma in a sterile precipiting tube and identify as above.
- Repeat this procedure for each bouillon culture.
- Incubate 4 to 6 hours at 37°C ± 1°C.
- Check for the presence of coagulum or examine the tube after 24 hours □ 2 hours of incubation.

8.2.3. Results

Coagulase is considered positive when it occupies ¾ of the initial volume of the liquid.

8.2.3.1. General case

The dishes contain a maximum of 150 characteristic and/or non-characteristic colonies.
Method of calculation:

- Number of Staphylococci with positive coagulase for each dish: a

$$a = \frac{b^c}{A^c} \times c^c + \frac{b^{nc}}{A^{nc}} \times c^{nc}$$

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A^c	is the number of subcultured characteristic colonies
A^{nc}	is the number of subcultured non-characteristic colonies;
b^c	is the number of characteristic colonies of positive coagulase Staphylococci;
b^{nc}	is the number of non-characteristic colonies of positive coagulase Staphylococci
c^c	is the total number of characteristic colonies of positive coagulase Staphylococci for the dish retained;
c^{nc}	is the total number of non-characteristic colonies of positive coagulase Staphylococci positive for the dish retained.

Round off the value obtained to the nearest whole number.

- Number of positive coagulase Staphylococci in test sample: N

The weighted average, calculated as follows from two successive retained dilutions:

$N = \frac{\sum a}{1,1 \times F} \times 10$ positive coagulase Staphylococci by g or ml

□a : sum of positive coagulase Staphylococci colonies identified on 2 retained dishes

F : rate of dilution corresponding to 1st retained dilution.

Expression of results:

- Round off the number N to the two largest whole digits
- Express to the tenth power

ex.:	Amount obtained	Amount rounded off	Result
	36364	36000	$3.6\ 10^4$

8.2.3.2. Estimation of small numbers:

If the dish inoculated with 0.1 ml of the first dilution retained for analysis contains less than 15 colonies, the result will be expressed as follows:

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$N = a\frac{1}{d} \times 10$ positive coagulase Staphylococci per g or ml

a : number of positive coagulase Staphylococci identified.

d : rate of dilution for the first dilution retained for analysis.

If the dish inoculated with 0.1 ml of the first dilution retained for analysis contains no positive coagulase Staphylococci the result shall be expressed as follows:

$N < \frac{1}{d} \times 10$ no positive coagulase Staphylococci per g or ml

d : Rate of dilution from the first dilution retained for analysis.

9. Coliform count by counting colonies obtained at 30°C

9.1. Principle

Inoculation in deeply in crystal violet and neutral red (VRBL) lactose bile agar was carried out in Petri dishes for each of the dilutions retained. After incubation for 24 hours at 30°C, the characteristic colonies that appeared were counted.

9.2. Apparatus and analytical conditions

Cultures are carried out in a sterile environment as ensured by a Bunsen burner.

- Plastic sterile Petri dishes with a diameter of 90 millimetres
- Sterile glass 16x160 cottoned test tubes
- Tube holder
- 2 ml plastic sterile pipettes graduated at 0.1 ml
- Water bath at 47°C ± 2°C
- Tube shaker
- Incubate at 30°C ± 1°C
- Incubate at 55°C ± 1°C
- Bunsen burner
- Colony counter

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9.3. Reagents

- Sterile diluent for decimal dilutions: tryptone salt (TS)
- 16x160 sterile tubes pre-filled with 9ml of sterile TS
- Cooled crystal violet and neutral red lactose bile agar (VRBL).

9.4. Procedure

9.4.1. Agar medium

- Once prepared, keep the VRBL agar cooled in the water bath at 47°C ± 2°C (for immediate usage).
- Never use a culture medium at a temperature higher than 50°C.
- Do not cool over 8 hours.
- For a deferred usage, keep agar cooled in an incubator at 55°C ±1°C.
- Melted culture mediums unused within 8 hours, shall never re-solidify for later usage.

9.4.2. Culture

- Homogenise each dilution before inoculation in Petri dishes prior to carrying out decimal dilutions.
- Transfer 1 ml of reference solution and/or decimal dilutions retained in respective Petri dishes. Change pipettes after each dilution.
- Introduce up to 20 minutes after the inoculum 15 to 20 ml of VRBL maintained in the water bath at 47°C ± 2°C.
- Slowly homogenise by shaking.
- Allow to solidify on laboratory bench (lid upwards).
- Pour approximately 5 ml of VRBL maintained in the water bath at 47°C ± 2°C.
- Allow to solidify on the laboratory bench (lid upwards).

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- Turn over dishes and incubate immediately 24 hours □ 2 hours at 30°C □ 1°C.

9.4.3. Count

Dishes containing less than 150 characteristic or non-characteristic colonies based on two successive dilutions are retained, but one of them must contain at least 15 characteristic colonies.

If only the dish inoculated with 1 ml of the 1st dilution contains under 15 characteristic colonies, then the dish will be retained for counting.

Characteristic colonies are counted manually or by using a counter.

Characteristic colonies after 24 hours □ 2 hours of incubation

- violet colonies sometimes surrounded by a red area (bile precipitation)
- diameter □ 0.5 mm

9.5. Results

9.5.1. General case

Dishes contain less than 150 characteristic or non-characteristic colonies based on two successive dilutions with one including at least 15 characteristic colonies.

Method of calculation:

Number N of micro-organisms counted at 30°C per millilitre (ml) or by gram (g) of product is obtained by calculating the weighted average of the 2 retained dishes.

$$N = \frac{\sum c}{1{,}1d}$$

□c : sum of characteristic colonies counted on the 2 retained dishes

d : dilution rate corresponding to the 1st dilution

Expression of results:

- round off the number N to the 2 largest digits
- express to the tenth power

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ex: 1.6 10^3 / g or ml

9.5.2. Estimation of small numbers

If the dish inoculated with 1 ml of the 1st dilution retained for analysis contains less than 15 characteristic colonies, the result will be expressed as follows:

$$N = c\frac{1}{d}$$

- c : sum of characteristic colonies counted
- d : rate of dilution

If the dish inoculated with 1ml of the 1st dilution retained for analysis contains no colonies then the result will be expressed as follows:

$N =< 1\frac{1}{d}$ micro-organisms per g or ml

d : rate of dilution.

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[1] Point to be studied at a later date by the sub-commission of methods of analysis and appraisal of wine.

[2] Point to be studied at a later date by the expert group “Wine microbiology”.

[3] Point to be studied at a later date by the expert group “Wine microbiology”.

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