

Microbiological Examination Methods of Food and Water

A Laboratory Manual

2nd Edition

Neusely da Silva
Marta H. Taniwaki
Valéria C.A. Junqueira
Neliane F.A. Silveira
Margarete M. Okazaki
Renato A.R. Gomes



MICROBIOLOGICAL EXAMINATION METHODS OF FOOD AND WATER



Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

MICROBIOLOGICAL EXAMINATION METHODS OF FOOD AND WATER

A Laboratory Manual

SECOND EDITION

NEUSELY DA SILVA, MARTA HIROMI TANIWAKI,
VALÉRIA CHRISTINA AMSTALDEN JUNQUEIRA,
NELIANE FERRAZ DE ARRUDA SILVEIRA,
MARGARETE MIDORI OKAZAKI &
RENATO ABEILAR ROMEIRO GOMES

Institute of Food Technology – ITAL, Campinas, SP, Brazil



CRC Press

Taylor & Francis Group

Boca Raton London New York Leiden

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

A BALKEMA BOOK

Originally published in Portuguese as: 'Manual de Metodos de Análise Microbiológica de Alimentos e Água ©2010, Livraria Varela, Sao Paulo, Brazil

English edition 'Microbiological Examination Methods of Food and Water: A Laboratory Manual', CRC Press/Balkema, Taylor & Francis Group, an informa business ©2013 Taylor & Francis Group, London, UK

Translation to English: Paul van Dender†

CRC Press/Balkema is an imprint of the Taylor & Francis Group, an informa business

© 2019 Taylor & Francis Group, London, UK

Typeset by Apex CoVantage, LLC

All rights reserved. No part of this publication or the information contained herein may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, by photocopying, recording or otherwise, without written prior permission from the publisher.

Although all care is taken to ensure integrity and the quality of this publication and the information herein, no responsibility is assumed by the publishers nor the author for any damage to the property or persons as a result of operation or use of this publication and/or the information contained herein.

Published by: CRC Press/Balkema
Schipholweg 107c, 2316 XC Leiden, The Netherlands
e-mail: Pub.NL@taylorandfrancis.com
www.crcpress.com – www.taylorandfrancis.com

Library of Congress Cataloging-in-Publication Data

Names: Silva, Neusely da., author.

Title: Microbiological examination methods of food and water : a laboratory manual / Neusely da Silva, Marta Hiromi Taniwaki, Valeria Christina Amstalden Junqueira, Neliane Ferraz de Arruda Silveira, Margarete Midori Okazaki & Renato Abeilar Romeiro Gomes.

Other titles: Manual de metodos de analise microbiologica de alimentos e agua. English

Description: Second edition. | Leiden, The Netherlands ; Boca Raton : CRC Press/Balkema, [2018] | Includes bibliographical references and index.

Identifiers: LCCN 2018034220 (print) | LCCN 2018035352 (ebook) | ISBN 9781315165011 (ebook) |

ISBN 9781138091887 (hardcover : alk. paper) | ISBN 9781138057111 (pbk. : alk. paper)

Subjects: MESH: Bacteriological Techniques—methods | Food Microbiology—methods | Water Microbiology | Laboratory Manuals

Classification: LCC QR115 (ebook) | LCC QR115 (print) | NLM QW 25.5.B2 | DDC 579/.16—dc23

LC record available at <https://lcn.loc.gov/2018034220>

ISBN: 978-1-138-05711-1 (Pbk)

ISBN: 978-1-138-09188-7 (Hbk)

ISBN: 978-1-315-16501-1 (eBook)

Table of contents

About the authors	XXVII
Preface	XXIX
List of tables	XXXI
List of figures	XXXV
1 Sampling, transport and storage of samples for analysis	1
Revision history	1
1.1 Introduction	1
1.1.1 Lot	1
1.1.2 Lot sample and sample unit	1
1.1.3 Lot sampling plans	2
1.1.4 Analytical unit	2
1.2 Collecting samples for analysis	2
1.2.1 Selection and preparation of containers for the sampling of foods contained in non-individual packages	3
1.2.2 Procedures for the sampling of foods contained in non-individual packages	3
1.2.3 Sampling of foods involved in foodborne diseases	4
1.2.4 Sampling of water	4
1.3 Transportation and storage of samples until analysis	5
1.3.1 Foods with low water activity	5
1.3.2 Frozen foods	5
1.3.3 Refrigerated foods	5
1.3.4 Commercially sterile foods in sealed packages	6
1.3.5 Water samples	6
1.4 References	7
2 Preparation of samples for analysis	9
Revision history	9
2.1 Introduction	9
2.2 Homogenization of samples and withdrawal of the analytical unit	10
2.2.1 Procedure for homogenization and withdrawal of analytical units from liquid products	11
2.2.2 Procedure for homogenization and withdrawal of analytical units from solid or concentrated liquid products	11
2.2.3 Procedure for withdrawing the analytical unit using the surface swabbing technique	12
2.2.3.1 Swab sampling	12
2.2.3.2 Sponge swab sampling	13
2.2.4 Procedure for withdrawing the analytical unit using the surface washing technique	13
2.2.4.1 Procedure for washing poultry carcasses	13
2.2.4.2 Procedure for washing other foods	13
2.2.4.3 Procedure for washing packages	14
2.2.5 Keeping of counter-samples	14
2.3 Preparation of the first dilution of the analytical unit	14

VI Table of contents

2.3.1	Diluents for presence/absence tests	14
2.3.2	Diluents for tests requiring differentiated handling of the sample	14
2.3.3	Diluents for general quantification tests	14
2.3.4	How to prepare an initial 1:10 (10^{-1}) dilution	15
2.3.5	How to prepare an initial dilution different from 1:10	16
2.3.6	Procedure for the preparation of the first dilution of liquid samples	16
2.3.7	Procedure for the preparation of the first dilution of solid or concentrated liquid samples	16
2.3.8	Procedure for the preparation of the first dilution of samples obtained by surface swabbing or surface washing	16
2.4	Serial decimal dilution of the sample	16
2.5	References	17
Annex 2.1	– Procedures for homogenizing the content and withdrawal of the analytical unit of different types of foods	18
a)	Powdered products	18
b)	Pasty or ground products	18
c)	Yogurts with fruit pieces	18
d)	Cheeses	18
e)	Very hard food products	18
f)	Pieces of solid foods	18
g)	Eggs in the shell	18
h)	Meat cuts for analysis of non-surface contamination	19
i)	Bivalves	19
j)	Gastropods	19
k)	Whole and sliced cephalopods	19
l)	Whole crustaceans such as crabs	19
m)	Sea urchins	19
n)	Whole fresh fish	19
Annex 2.2	– Special cases in which there are variations in the analytical unit and/or dilution and/or diluents recommended for the preparation of the first dilution of samples of different types of foods	19
a)	Liquids with low levels of contamination	19
b)	Fatty foods	20
c)	Lump-forming powders	20
d)	Thickeners or products containing natural antimicrobial compounds	20
e)	Gelatin	20
f)	Acid products	20
g)	Fine flours or meals, cereal grains, animal feed	21
h)	Cocoa and chocolate	21
i)	Egg white	21
j)	Fermented products containing live microorganisms intended for the quantification of the contaminating microflora (except probiotics)	21
k)	Powdered dairy products (dried milk, dried sweet whey, dried acid whey, dried buttermilk, lactose)	21
l)	Butter	21
m)	Frozen dairy products and ice cream	21
n)	Cheeses	22
o)	Fermented dairy products	22
p)	Casein and caseinates	22
q)	Rennet casein difficult to dissolve	22

r)	Powdered milk-based infant foods	22
s)	Custard, desserts and sweet cream (pH > 5)	23
t)	Mollusks (bivalves and gastropods) and sea urchins	23
u)	Sea cucumbers (<i>Holothuroidea</i>) and tunicates	23
3	Basic plate count techniques for enumeration of microorganisms	25
	Revision history	25
3.1	Introduction	25
3.2	Pour plate technique	26
3.2.1	Material required for the analyses	26
3.2.2	Procedure	26
3.3	Spread plate technique	27
3.3.1	Material required for the analyses	28
3.3.2	Procedure	28
3.4	Drop plate technique	29
3.4.1	Material required for the analyses	29
3.4.2	Procedure	29
3.5	Membrane filtration	30
3.5.1	Material required for the analyses	30
3.5.2	Procedure	30
3.6	Counting colonies and calculating results according to APHA	31
3.6.1	Pour plate calculations	31
3.6.1.1	Rules for calculating the pour plate results in the standard situation	32
3.6.1.2	Rules for calculating the pour plate results in unusual situations	33
3.6.1.3	Calculating the pour plates results for samples prepared by the surface swabbing technique (swabs or sponges)	35
3.6.1.4	Calculating the pour plate results of samples prepared by the surface washing technique	36
3.6.2	Spread plate calculations	36
3.6.3	Drop plate calculations	36
3.6.4	Membrane filtration calculations	36
3.7	(revised) Counting colonies and calculating results according to ISO 7218:2007/Amd.1:2013	37
3.7.1	(new) General requirements for the calculation of results	37
a)	Number of Petri dishes per dilution	37
b)	Maximum and minimum acceptable number of colonies on counting plates	37
c)	Decimal dilution of the number of colonies	38
d)	Acceptable variation between counts of the pair of plates of a duplicate	38
e)	Presentation of the result	38
3.7.2	General rules for the calculation of results	38
3.7.3	Rules for calculation in unusual situations	41
3.8	References	43
Annex 3.1	– (new) Limits of agreement for sums of colony counts of two parallel Petri dishes or colony counts from one Petri dish per dilution step over two 10-fold dilution steps (with a probability of 99% per comparison) (ISO 14461-2:2005)	44
Annex 3.2	– (new) Limits of agreement for colony counts of two parallel Petri dishes (with a probability of 99% per comparison) (ISO 14461-2:2005)	45
4	Basic techniques for microbial enumeration by the most probable number (MPN) method	47
	Revision history	47
4.1	Introduction	47

VIII Table of contents

4.2	Multiple dilution test	48
4.2.1	Material required for the analyses	49
4.2.2	Procedure	49
4.3	Single dilution test	50
4.3.1	Material required for the analyses	50
4.3.2	Procedure	50
4.4	Calculation of the results	51
4.4.1	Calculating the results of the multiple dilution test	51
4.4.1.1	Calculation using the MPN tables (for decimal dilutions)	51
4.4.1.2	Calculating using the Thomas formula (for non-decimal dilutions)	52
4.4.1.3	Calculating the results of the samples prepared by the surface swabbing or surface washing techniques	52
4.4.2	Calculating the results of the single dilution test	53
4.4.2.1	Rules for calculations performed using Table MPN-3	53
4.4.2.2	Calculation for samples prepared by the surface swabbing or surface washing techniques	53
4.5	References	53
	Annex 4.1 – MPN tables	54
5	Basic techniques for the detection of the presence/absence of microorganisms	57
	Revision history	57
5.1	Introduction	57
5.1.1	Enrichment	57
5.1.1.1	Pre-enrichment	58
5.1.1.2	Selective enrichment	58
5.1.2	Isolation in solid media (selective differential plating)	58
5.1.3	Confirmation	59
5.1.3.1	Catalase test	59
5.1.3.2	Citrate test	59
5.1.3.3	Amino acid decarboxylation tests	59
5.1.3.4	Phenylalanine deaminase test	59
5.1.3.5	Carbohydrate fermentation tests	59
5.1.3.6	Indole test	60
5.1.3.7	Malonate test	60
5.1.3.8	Oxidation/fermentation (O/F) test	60
5.1.3.9	Oxidase test	61
5.1.3.10	Nitrate reduction test	61
5.1.3.11	Urease test	61
5.1.3.12	Methyl red (MR) test	61
5.1.3.13	Voges-Proskauer (VP) test	62
5.2	Material required for the analyses	62
5.3	Procedure	62
5.3.1	Pre-enrichment	62
5.3.2	Selective enrichment	62
5.3.3	Differential plating	62
5.3.3.1	Streak plating technique for obtaining pure cultures	62
5.3.4	Selection of colonies and subculturing of cultures for confirmation	63
5.3.4.1	Technique for the subculturing of pure cultures starting from colonies isolated from plates	63

5.3.5	Confirmation tests	63
5.3.5.1	Gram staining (Hucker's method)	63
5.3.5.2	(new) KOH test for confirmation of doubtful Gram staining (Gregersen, 1978)	64
5.3.5.3	Spore staining (Schaeffer-Fulton's method)	64
5.3.5.4	Spore staining (Ashby's method)	64
5.3.5.5	Wet mounts for direct (fresh) microscopic observation	64
5.4	References	64
6	Aerobic plate count	65
	Revision history	65
6.1	Introduction	65
6.1.1	The importance and significance of the total aerobic mesophilic count	65
6.1.2	Definition of psychrotrophs	66
6.1.3	Comments on methods of analysis	67
6.2	(revised) Plate count method APHA 8:2015 for aerobic mesophilic bacteria in foods and water	69
6.2.1	Material required for analysis	69
6.2.2	Procedure	70
6.2.2.1	Pour plate technique	70
6.2.2.2	Spread plate technique	71
6.2.2.3	Membrane filtration technique	71
6.3	(revised) Petrifilm™ AOAC official methods for aerobic mesophilic bacteria in foods	73
6.3.1	Material required for analysis	73
6.3.2	Procedure	73
6.4	(revised) Plate count method APHA 13.61:2015 for aerobic psychrotrophic bacteria in foods	73
6.4.1	Material required for analysis	74
6.4.2	Procedure	74
6.5	(new) Plate count methods ISO 4833-1:2013 and ISO 4833-2:2013/Corr.1:2014 for aerobic mesophilic bacteria in foods	75
6.5.1	Material required for analysis	75
6.5.2	Procedure	75
6.6	(new) Plate count method BAM/FDA:2001 for aerobic mesophilic bacteria in foods	77
6.6.1	Material required for analysis	77
6.6.2	Procedure	77
6.7	References	79
7	Yeasts and molds	81
	Revision history	81
7.1	Introduction	81
7.1.1	Yeasts and molds in foods	81
7.1.2	Comments on methods of analysis for total yeast and mold counts	82
7.1.3	Psychrotrophic fungi	82
7.1.4	Heat-resistant molds	84
7.1.5	Preservative-resistant yeasts (PRY)	84
7.1.5.1	<i>Zigosaccharomyces bailii</i>	85
7.1.5.2	<i>Zygosaccharomyces bisporus</i>	85
7.1.5.3	<i>Schizosaccharomyces pombe</i>	85

X Table of contents

	7.1.5.4	<i>Candida krusei</i>	85
	7.1.5.5	<i>Pichia membranaefaciens</i>	86
7.1.6		Osmophilic yeasts	86
	7.1.6.1	<i>Zygosaccharomyces rouxii</i>	86
7.1.7		Direct plating method ICFM for particulate foods	87
7.2.A		Plate count method APHA 21:2015 for yeasts and molds in foods	87
	7.2.A.1	Material required for analysis	87
	7.2.A.2	Procedure	87
7.2.B		(new) Plate count methods ISO 21527-1:2008 and ISO 21527-2:2008 for yeasts and molds in foods	90
	7.2.B.1	Material required for analysis	90
	7.2.B.2	Procedure	90
7.2.C		(new) Plate count method BAM/FDA:2001 for yeasts and molds in foods	92
	7.2.C.1	Material required for analysis	92
	7.2.C.2	Procedure	92
7.3		(revised) Plate count method APHA 13:2015 for psychrotrophic fungi in foods	93
	7.3.1	Material required for analysis	93
	7.3.2	Procedure	93
7.4		(revised) Plate count method APHA 22.4:2015 for heat-resistant molds in foods	95
	7.4.1	Material required for analysis	95
	7.4.2	Procedure	95
7.5		Pitt and Hocking (2009) methods for preservative-resistant yeasts in foods	97
	7.5.1	Material required for analysis	97
	7.5.2	Procedure	97
	7.5.2.1	Presence/absence method	98
	7.5.2.2	Direct plate count method	99
7.6		(revised) Membrane filtration or plate count methods APHA 17.3:2015 for osmophilic yeasts in foods	99
	7.6.1	Material required for analysis	99
	7.6.2	Procedure	99
	7.6.2.1	Membrane filtration method	99
	7.6.2.2	Plate count method	100
7.7		(new) Direct plating method Hocking <i>et al.</i> (2006) for percentage of fungal infection in particulate foods	100
	7.7.1	Material required for analysis	101
	7.7.2	Procedure	101
7.8		References	101
8		<i>Enterobacteriaceae</i>	103
		Revision history	103
8.1		Introduction	103
	8.1.1	Taxonomy	103
	8.1.2	Comments on methods of analysis	104
8.2		(revised) Plate count method APHA 9.62:2015 for <i>Enterobacteriaceae</i> in foods	105
	8.2.1	Material required for analysis	105
	8.2.2	Procedure	105
8.3		(revised) Presence/absence (P/A) or most probable number (MPN) method APHA 9.61:2015 for <i>Enterobacteriaceae</i> in foods	106
	8.3.1	Material required for analysis	106
	8.3.2	Procedure	106

8.4	(revised) AOAC official method 2003.1 (Petrifilm™) for <i>Enterobacteriaceae</i> in selected foods	108
8.4.1	Material required for analysis	108
8.4.2	Procedure	108
8.5	(new) Plate count method ISO 21528-2:2017 for <i>Enterobacteriaceae</i> in foods	109
8.5.1	Material required for analysis	109
8.5.2	Procedure	109
8.6	(new) Presence/absence (P/A) or most probable number (MPN) method ISO 21528-1:2017 for <i>Enterobacteriaceae</i> in foods	111
8.6.1	Material required for analysis	111
8.6.2	Procedure	111
8.7	References	113
9	Total and thermotolerant coliforms and <i>Escherichia coli</i>	115
	Revision history	115
9.1	Introduction	115
9.1.1	Definition of total coliforms	115
9.1.2	Definition of thermotolerant coliforms	116
9.1.3	<i>E. coli</i>	116
9.1.4	Use as indicators	116
9.1.5	Comments on methods of analysis	117
9.2	(revised) Most probable number (MPN) method APHA 9:2015 for total/thermotolerant coliforms and <i>E. coli</i> in foods	119
9.2.1	Material required for analysis	119
9.2.2	Procedure	120
9.3	Most probable number (MPN) methods ISO 4831:2006 and ISO 7251:2005 for total coliforms and presumptive <i>E. coli</i> in foods	123
9.3.1	Material required for analysis	123
9.3.2	Procedure	123
9.4	(revised) Most probable number (MPN) method APHA/AWWA/WEF:2012 for total and thermotolerant coliforms and <i>E. coli</i> in water	126
9.4.1	Material required for analysis	126
9.4.2	Procedure	126
9.5	(new) Most probable number (MPN) method BAM/FDA:2017 for total/thermotolerant coliforms and <i>E. coli</i> in foods	128
9.5.1	Material required for analysis	128
9.5.2	Procedure	129
9.6	(revised) Plate count method APHA:2015 for total coliforms in foods	130
9.6.1	Material required for analysis	130
9.6.2	Procedure	130
9.7	(new) Membrane filtration method ISO 9308-1:2014/Amd.1:2016 for total coliforms and <i>E. coli</i> in water	132
9.7.1	Material required for analysis	132
9.7.2	Procedure	132
9.8	References	134
10	<i>Staphylococcus aureus</i>	135
	Revision history	135
10.1	Introduction	135
10.1.1	Taxonomy	135
10.1.1.1	The genus <i>Staphylococcus</i>	135

XII Table of contents

10.1.1.2	The coagulase-positive staphylococci	136
10.1.1.3	<i>Staphylococcus aureus</i>	137
10.1.2	Epidemiology	137
10.1.2.1	<i>Staphylococcus aureus</i> enterotoxins	137
10.1.2.2	Staphylococcal food poisoning	138
10.1.3	Comments on methods of analysis	139
10.2	(revised) Plate count method APHA 39.63:2015 for coagulase-positive staphylococci and <i>Staphylococcus aureus</i> in foods	140
10.2.1	Material required for analysis	140
10.2.2	Procedure	140
10.3	(revised) Most probable number (MPN) method APHA39.62:2015 for coagulase-positive staphylococci and <i>Staphylococcus aureus</i> in foods	143
10.3.1	Material required for analysis	143
10.3.2	Procedure	143
10.4	(revised) Presence/absence method APHA 39.61:2015 for coagulase-positive staphylococci and <i>Staphylococcus aureus</i> in foods	145
10.4.1	Material required for analysis	145
10.4.2	Procedure	145
10.5	References	147
11	<i>Bacillus cereus</i>	149
	Revision history	149
11.1	Introduction	149
11.1.1	Taxonomy	149
11.1.1.1	<i>Bacillus cereus</i> group	149
	<i>Bacillus anthracis</i>	150
	<i>Bacillus thuringiensis</i>	150
	<i>Bacillus mycoides</i>	150
	<i>Bacillus pseudomycooides</i>	151
	<i>Bacillus weihenstephanensis</i>	151
	<i>Bacillus cytotoxicus</i>	151
	New species	151
11.1.1.2	<i>Bacillus cereus</i>	151
11.1.2	Epidemiology	152
11.1.3	Comments on methods of analysis	152
11.2	(revised) Plate count method APHA 31.61:2015 for <i>Bacillus cereus</i> in foods	153
11.2.1	Material required for analysis	153
11.2.2	Procedure	154
11.3	(revised) Most probable number (MPN) method APHA 31.62:2015 for <i>Bacillus cereus</i> in foods	157
11.3.1	Material required for analysis	157
11.3.2	Procedure	157
11.4	References	159
12	<i>Clostridium perfringens</i>	161
	Revision history	161
12.1	Introduction	161
12.1.1	Taxonomy	161
12.1.2	Epidemiology	162
12.1.2.1	<i>Clostridium perfringens</i> type A food poisoning	162
12.1.2.2	<i>Clostridium perfringens</i> type C necrotic enteritis	163
12.1.3	Comments on methods of analysis	163

12.2	(revised) Plate count method APHA 33.72:2015 for <i>Clostridium perfringens</i> in foods	164
12.2.1	Material required for analysis	164
12.2.2	Procedure	165
12.3	(revised) Presence/absence method APHA 33.71:2015 for <i>Clostridium perfringens</i> in foods	167
12.3.1	Material required for analysis	167
12.3.2	Procedure	167
12.4	(new) Plate count method BAM/FDA:2001 for <i>Clostridium perfringens</i> in foods	169
12.4.1	Material required for analysis	169
12.4.2	Procedure	169
12.5	(new) Plate count method ISO 7937:2004 for <i>Clostridium perfringens</i> in foods	171
12.5.1	Material required for analysis	171
12.5.2	Procedure	172
12.6	(new) Membrane filtration method ISO 14189:2013 for <i>Clostridium perfringens</i> in water	173
12.6.1	Material required for analysis	173
12.6.2	Procedure	173
12.7	References	175
13	Enterococci	177
	Revision history	177
13.1	Introduction	177
13.1.1	Taxonomy	177
13.1.1.1	Enterococci	178
	Description of the genus <i>Enterococcus</i>	178
13.1.1.2	Fecal streptococci	179
	Description of the genus <i>Streptococcus</i>	180
13.1.1.3	Differentiation of enterococci from group bovis fecal streptococci	180
13.1.2	Comments on methods of analysis	180
13.2	(revised) Plate count method APHA 10.5:2015 for enterococci and fecal streptococci in foods	181
13.2.1	Material required for analysis	181
13.2.2	Procedure	182
13.3	(revised) Most probable number (MPN) method APHA 10.2:2015 for enterococci and fecal streptococci in foods	183
13.3.1	Material required for analysis	183
13.3.2	Procedure	183
13.4	(revised) Membrane filtration method APHA/AWWA/WEF 9230C.3c:2012 for enterococci and fecal streptococci in water	184
13.4.1	Material required for analysis	184
13.4.2	Procedure	184
13.5	Membrane filtration method ISO 7899-2:2000 for intestinal enterococci in water	186
13.5.1	Material required for analysis	186
13.5.2	Procedure	186
13.6	References	186
14	Lactic acid bacteria	189
	Revision history	189
14.1	Introduction	189
14.1.1	Carnobacterium	189
14.1.2	Enterococcus	191
14.1.3	Fructobacillus	191

14.1.4	Lactobacillus	192
14.1.5	Lactococcus	192
14.1.6	Leuconostoc	193
14.1.7	Oenococcus	193
14.1.8	Pediococcus	194
14.1.9	Streptococcus	194
14.1.10	Tetragenococcus	195
14.1.11	Weissella	195
14.1.12	Comments on methods of analysis	195
14.2	(revised) Plate count method APHA 19.52:2015 for lactic acid bacteria in foods	198
14.2.1	Material required for analysis	198
14.2.2	Procedure	198
14.3	(revised) MPN methods APHA 19.526:2015 and APHA 19.524:2015 for lactic acid bacteria in foods	200
14.3.1	Material required for analysis	200
14.3.2	Procedure using MRS broth	200
14.3.3	Procedure using Rogosa SL broth	202
14.4	(new) Plate count method ISO 15214:1998 for lactic acid bacteria in foods	202
14.4.1	Material required for analysis	202
14.4.2	Procedure	202
14.5	References	205
15	<i>Campylobacter</i>	207
	Revision history	207
15.1	Introduction	207
15.1.1	Taxonomy	207
15.1.1.1	Campylobacter	207
15.1.1.2	Thermotolerant Campylobacter	209
15.1.2	Epidemiology	209
15.2	(revised) Presence/absence method ISO 10272-1:2017 for thermotolerant <i>Campylobacter</i> in foods	210
15.2.1	Material required for analysis	210
15.2.2	Procedure	210
15.3	(new) Plate count method ISO 10272-2:2017 for thermotolerant <i>Campylobacter</i> in foods	213
15.3.1	Material required for analysis	214
15.3.2	Procedure	214
15.4	References	216
16	<i>Cronobacter</i>	217
	Revision history	217
16.1	Introduction	217
16.1.1	Taxonomy	217
	Cronobacter Iversen <i>et al.</i> (2008)	218
16.1.2	Epidemiology	219
16.1.3	Codex Alimentarius microbiological criteria for Cronobacter spp. in powdered infant formulae	219
16.1.4	Comments on methods of analysis	220
16.2	(revised) Presence/absence method ISO 22964:2017 for <i>Cronobacter</i> in foods	220
16.2.1	Material required for analysis	220
16.2.2	Procedure	221

16.3	(new) Presence/absence method BAM/FDA:2012 for <i>Cronobacter</i> in dehydrated powdered infant formula	223
16.3.1	Material required for analysis	223
16.3.2	Procedure	223
16.4	References	225
17	<i>Pseudomonas</i>	227
	Revision history	227
17.1	Introduction	227
17.1.1	Taxonomy	227
17.1.1.1	<i>Pseudomonas</i>	227
	<i>Pseudomonas</i> in treated water intended for human consumption	229
	<i>Pseudomonas</i> in mineral water and natural water	230
	<i>Pseudomonas</i> in foods	230
17.1.1.2	<i>Shewanella</i>	230
	<i>Shewanella putrefaciens</i> (synonym <i>Pseudomonas putrefaciens</i>)	231
17.1.1.3	<i>Janthinobacterium</i>	231
	<i>Janthinobacterium lividum</i> (synonym <i>Pseudomonas mephitica</i>)	232
17.1.1.4	<i>Stenotrophomonas</i>	232
	<i>Stenotrophomonas maltophilia</i> (synonym <i>Pseudomonas maltophilia</i>)	232
17.2	(revised) MPN method APHA/AWWA/WEF 9213:2012 for <i>Pseudomonas aeruginosa</i> in water	233
17.2.1	Material required for analysis	233
17.2.2	Procedure	233
17.3	Membrane filtration method ISO 16266:2006 for <i>Pseudomonas aeruginosa</i> in water	234
17.3.1	Material required for analysis	234
17.3.2	Procedure	234
17.4	Plate count method ISO 13720:2010 for presumptive <i>Pseudomonas</i> spp. in meat and meat products	237
17.4.1	Material required for analysis	237
17.4.2	Procedure	237
17.5	Plate count method ISO 11059:2009 for <i>Pseudomonas</i> spp. in milk and milk products	237
17.5.1	Material required for analysis	237
17.5.2	Procedure	238
17.6	References	240
18	<i>Listeria monocytogenes</i>	243
	Revision history	243
18.1	Introduction	244
18.1.1	Taxonomy	244
18.1.2	Epidemiology	246
18.1.3	Comments on methods of analysis	246
18.2	(revised) Presence/absence or MPN method BAM/FDA:2017 for <i>Listeria monocytogenes</i> in foods	248
18.2.1	Material required for analysis	248
18.2.2	Procedure	248
	18.2.2.1 Presence/absence test and MPN count	250
	18.2.2.2 Direct plate count	253
18.3	(revised) Presence/absence method USDA/MLG:2017 for <i>Listeria monocytogenes</i> in foods	254
18.3.1	Material required for analysis	254
18.3.2	Procedure	254

18.4	(revised) Plate count method ISO 11290-2:2017 for <i>Listeria</i> spp. and <i>Listeria monocytogenes</i> in foods	257
18.4.1	Material required for analysis	257
18.4.2	Procedure	257
18.5	(revised) Presence/absence method ISO 11290-1:2017 for <i>Listeria</i> spp. and <i>Listeria monocytogenes</i> in foods	260
18.5.1	Material required for analysis	260
18.5.2	Procedure	260
18.6	References	262
19	<i>Salmonella</i>	265
	Revision history	265
19.1	Introduction	266
19.1.1	Taxonomic classification of <i>Salmonella</i>	266
19.1.2	Serological classification of <i>Salmonella</i>	268
	Somatic (“O”) antigens	268
	Capsular (surface or envelope) antigens	268
	Flagellar (“H”) antigens	269
	The White-Kauffmann-Le Minor system	269
	<i>Salmonella</i> serovar nomenclature	269
	Serovars most commonly found	269
19.1.3	Biochemical characteristics of <i>Salmonella</i>	270
19.1.4	Epidemiology	270
19.1.5	Comments on traditional methods used for the examination of <i>Salmonella</i>	272
19.1.6	Comments on alternative methods for the analysis of <i>Salmonella</i>	274
19.1.7	Composite samples for analysis	274
19.2	(revised) Presence/absence method ISO 6579-1:2017 for <i>Salmonella</i> in foods	274
19.2.1	Material required for analysis	277
19.2.2	Procedure	277
19.3	(revised) Presence/absence method BAM/FDA:2018 for <i>Salmonella</i> in foods	282
19.3.1	Material required for analysis	282
19.3.2	Procedure	282
19.4	(revised) Presence/absence method MLG/USDA:2017 for <i>Salmonella</i> in foods	292
19.4.1	Material required for analysis	292
19.4.2	Procedure	292
19.5	References	296
20	<i>Vibrio cholerae</i> and <i>Vibrio parahaemolyticus</i>	299
	Revision history	299
20.1	Introduction	300
20.1.1	Taxonomy	300
20.1.2	Epidemiology	305
	20.1.2.1 <i>V. cholerae</i>	305
	20.1.2.2 <i>V. parahaemolyticus</i>	305
	20.1.2.3 <i>V. vulnificus</i>	305
20.1.3	Comments on methods of analysis	306
20.2.A	Presence/absence method BAM/FDA:2004 for <i>Vibrio cholerae</i> in foods	307
20.2.A.1	Material required for analysis	307
20.2.A.2	Procedure	307

20.2.B (revised) Presence/absence and MPN methods APHA 40.61:2015 for <i>Vibrio cholerae</i> in foods and water	309
20.2.B.1 Material required for analysis	310
20.2.B.2 Procedure	310
20.3.A MPN method BAM/FDA:2004 for <i>Vibrio parahaemolyticus</i> in foods	313
20.3.A.1 Material required for analysis	313
20.3.A.2 Procedure	313
20.3.B (revised) Presence/absence or MPN method APHA 40.62/40.63:2015 for <i>Vibrio</i> <i>parahaemolyticus</i> and <i>Vibrio vulnificus</i> in foods	315
20.3.B.1 Material required for analysis	315
20.3.B.2 Procedure	316
20.4 (revised) Presence/absence method ISO 21872-1:2017 for potentially enteropathogenic <i>Vibrio cholerae</i> and <i>Vibrio parahaemolyticus</i> in foods	318
20.4.1 Material required for analysis	318
20.4.2 Procedure	318
20.5 References	322
21 <i>Yersinia enterocolitica</i>	325
Revision history	325
21.1 Introduction	325
21.1.1 Taxonomy	325
21.1.2 Epidemiology	327
21.2 Presence/absence method ISO 10273:2017 for pathogenic <i>Yersinia enterocolitica</i> in foods	328
21.2.1 Material required for analysis	328
21.2.2 Procedure	328
21.3 References	332
22 Bacterial spore count	333
Revision history	333
22.1 Introduction	333
22.1.1 The bacterial spore	333
22.1.1.1 Sequence of spore formation	333
22.1.1.2 Spore ultrastructure	334
22.1.1.3 Mechanisms of spore resistance	334
22.1.2 Taxonomy of spore-forming bacteria important in foods	335
22.1.2.1 <i>Aeribacillus</i>	335
22.1.2.2 <i>Alicyclobacillus</i>	335
<i>Alicyclobacillus acidoterrestris</i>	336
<i>Alicyclobacillus acidocaldarius</i>	336
<i>Alicyclobacillus acidiphilus</i>	336
<i>Alicyclobacillus contaminans</i>	336
<i>Alicyclobacillus dauci</i>	336
<i>Alicyclobacillus fastidiosus</i>	337
<i>Alicyclobacillus herbarius</i>	337
<i>Alicyclobacillus pomorum</i>	337
<i>Alicyclobacillus sacchari</i>	337
22.1.2.3 <i>Aneurinibacillus</i>	337
<i>Aneurinibacillus thermoaerophilus</i>	338

XVIII Table of contents

22.1.2.4	<i>Anoxybacillus</i>	338
	<i>Anoxybacillus contaminans</i>	338
	<i>Anoxybacillus flavithermus</i>	338
	<i>Anoxybacillus tepidamans</i>	339
22.1.2.5	<i>Bacillus</i>	339
	<i>Bacillus coagulans</i>	339
	<i>Bacillus smithii</i>	340
	<i>Bacillus sporothermodurans</i>	340
22.1.2.6	<i>Brevibacillus</i>	341
22.1.2.7	<i>Clostridium</i>	341
	<i>Clostridium botulinum</i>	342
	Proteolytic clostridia	343
	Saccharolytic clostridia	343
	Psychrophilic and psychrotrophic clostridia that cause the spoilage of refrigerated vacuum-packed meats	344
22.1.2.8	<i>Cohnella</i>	344
22.1.2.9	<i>Desulfotomaculum</i>	345
	<i>Desulfotomaculum nigrificans</i>	345
22.1.2.10	<i>Fictibacillus</i>	345
	<i>Fictibacillus gelatini</i>	345
	<i>Fictibacillus nanhaiensis</i>	346
22.1.2.11	<i>Geobacillus</i>	346
	<i>Geobacillus stearothermophilus</i>	346
22.1.2.12	<i>Hathewayia</i>	347
22.1.2.13	<i>Jeotgalibacillus</i>	347
	<i>Jeotgalibacillus alimentarius</i>	347
22.1.2.14	<i>Lentibacillus</i>	347
22.1.2.15	<i>Lysinibacillus</i>	348
22.1.2.16	<i>Moorella</i>	348
22.1.2.17	<i>Oceanobacillus</i>	349
22.1.2.18	<i>Paenibacillus</i>	349
22.1.2.19	<i>Paraclostridium</i>	349
22.1.2.20	<i>Sporolactobacillus</i>	350
22.1.2.21	<i>Thermoanaerobacter</i>	350
22.1.2.22	<i>Thermoanaerobacterium</i>	350
	<i>T. thermosaccharolyticum</i>	350
22.1.2.23	<i>Virgibacillus</i>	351
22.2	(revised) Methods APHA 25:2015 and 26:2015 for spores of total and flat-sour thermophilic aerobic sporeformers in foods	351
22.2.1	Material required for analysis	352
22.2.2	Procedure for the analysis of sugar	352
22.2.3	Procedure for the analysis of starch	352
22.2.4	Procedure for the analysis of whole tomatoes, tomato pulp, tomato puree and concentrated milk	353
22.2.5	Procedure for the analysis of nonfat dry milk	353
22.2.6	Procedure for the analysis of milk cream	354
22.2.7	Procedure for the analysis of other foods and ingredients (general)	354
22.3	(revised) Methods APHA 27:2015 for spores of thermophilic anaerobic sporeformers in foods	356
22.3.1	Material required for analysis	356

22.3.2	Procedure for the analysis of sugar and powdered milk	356
22.3.3	Procedure for the analysis of starches and flours	357
22.3.4	Procedure for the analysis of cereals and alimentary pastes	357
22.3.5	Procedure for the analysis of fresh mushrooms	357
22.3.6	Procedure for the analysis of “in-process” products	357
22.4	(revised) Methods APHA 28:2015 for spores of sulfide spoilage anaerobic sporeformers in foods	357
22.4.1	Material required for analysis	357
22.4.2	Procedure for the analysis of sugar	357
22.4.3	Procedure for the analysis of starch and flour	358
22.4.4	Procedure for the analysis of skim milk powder	358
22.4.5	Procedure for the analysis of soy protein isolates	358
22.5	(revised) Methods APHA 23:2015 for spores of mesophilic aerobic sporeformers in foods	359
22.5.1	Material required for analysis	359
22.5.2	Procedure for foods in general	359
22.5.3	Procedure for the analysis of milk and dairy products	361
22.5.4	Procedure for the analysis of water	361
22.6	(revised) Methods APHA 24:2015 for spores of mesophilic anaerobic sporeformers in foods	361
22.6.1	Material required for analysis	362
22.6.2	Procedure for the analysis of sugar	362
22.6.3	Procedure for the analysis of starch, flours and other cereal products	362
22.6.4	Procedure for the analysis of dehydrated vegetables	363
22.6.5	Procedure for the analysis of seasonings and spices	363
22.6.6	Procedure for the analysis of egg powder, milk powder and other powdered dairy products	363
22.6.7	Procedure for the analysis of fluid milk and cheeses	363
22.6.8	Other procedures for mesophilic anaerobic sporeformers	364
22.7	Methods IFU 12:2007 for <i>Alicyclobacillus</i> in foods	364
22.7.1	Material required for analysis	364
22.7.2	Procedure for the analysis of raw material	365
22.7.3	Procedure for analysis of the finished product	366
22.7.4	Interpretation and calculation of the results	366
22.8	References	367
23	Commercial sterility	373
	Revision history	373
23.1	Introduction	373
23.1.1	Definition of commercial sterility	374
23.1.2	Classification of commercially sterile foods	374
23.1.3	Parameters for evaluating the heat resistance of microorganisms	374
	23.1.3.1 Survival curve and decimal reduction time (D value)	374
	23.1.3.2 Number of decimal reductions	376
	23.1.3.3 Thermal destruction curve and temperature coefficient (z value)	376
23.1.4	D and z values of microorganisms of importance in foods	377
	Vegetative cells	378
	Heat-resistant mold spores	378
	Bacterial spores	378
	Strictly thermophilic aerobic spore-forming bacteria	378
	Strictly thermophilic anaerobic spore-forming bacteria	378

	Facultative thermophilic aerobic spore-forming bacteria	378
	Mesophilic aerobic spore-forming bacteria	378
	Mesophilic anaerobic spore-forming bacteria	379
23.1.5	Dimensioning heat treatments and thermal processing	379
	23.1.5.1 Definition of the intensity of the thermal process	379
23.1.6	Microbial spoilage of canned foods	380
	23.1.6.1 Underprocessing	380
	23.1.6.2 Post process contamination (leakage)	380
	23.1.6.3 Spoilage by strictly thermophiles	380
	23.1.6.4 Microbial multiplication before heat treatment	381
	23.1.6.5 Non-microbial causes of spoilage	381
23.1.7	Useful terms	381
23.2	(revised) Method APHA:2015 for commercial sterility or cause of spoilage of low-acid canned foods	382
	23.2.1 Material required for analysis	382
	23.2.2 Procedure	382
	23.2.3 Interpretation of the results	386
23.3	(revised) Method APHA:2015 for commercial sterility for cause of spoilage of acid canned foods	389
	23.3.1 Material required for analysis	390
	23.3.2 Procedure	390
	23.3.3 Interpretation of the results	394
23.4	References	396
24	Guidelines on preparation of culture media	399
	Revision history	399
24.1	Introduction	399
	24.1.1 Ingredients used in the formulation of culture media	399
	24.1.1.1 (revised) Water for preparing media and reagents	399
	24.1.1.2 Nutrient sources for culture media	400
	Peptones	400
	Meat extract, yeast extract and malt extract	401
	Carbohydrates	402
	Minerals and essential metals	402
	24.1.1.3 Selective agents	402
	Antibiotics	402
	Bile and bile salts	402
	Chemical compounds	402
	24.1.1.4 Differential agents	402
	pH indicators	403
	Hydrogen sulfide (H ₂ S) indicators	403
	Other differential agents	403
	24.1.1.5 Reducing agents	403
	24.1.1.6 Buffering agents	403
	24.1.1.7 Chromogenic and fluorogenic substrates	403
	X-Glucuronide	404
	MUG	404
	ONPG	404
	Salmon-Gal	404
	X-Gal	404

	X-Glu	404
	X-Alpha-glicoside	404
	X-Phos-inositol	404
24.1.1.8	Agar	404
24.1.2	(revised) Types of culture media	404
	Chemically defined medium	404
	Chemically undefined medium or partially undefined medium	404
	Chromogenic or fluorogenic medium	405
	Liquid medium	405
	Solid and semisolid medium	405
	Transport medium	405
	Preservation medium	405
	Suspension medium (diluent)	405
	Resuscitation medium	405
	Pre-enrichment medium or enrichment medium	405
	Selective enrichment medium	405
	Non-selective enrichment medium	405
	Isolation medium	405
	Selective isolation medium	405
	Non-selective isolation medium	405
	Differential medium	405
	Identification medium	406
	Ready-to-use medium	406
	Medium prepared from commercially dehydrated formulations	406
	Medium prepared from individual components	406
24.2	Procedure for preparation of culture media	406
24.2.1	Storing supplies and ingredients for preparation of culture media	406
24.2.2	Weighing and rehydration	406
24.2.3	Dissolution and dispersion	407
24.2.4	Verification and adjustment of the pH before sterilization	407
24.2.5	Distribution	407
24.2.6	Sterilization by moist heat	407
24.2.7	Sterilization by filtration	408
24.2.8	Verification after sterilization	408
24.2.9	Preparation of supplements for culture media	409
24.2.10	Storage of sterilized media until the moment of use	409
	24.2.10.1 Recommendations from ISO 11133:2014	409
	24.2.10.2 (new) Recommendations from <i>Standard Methods for the Examination of Water and Wastewater</i> (Hunt, 2012)	409
24.2.11	(revised) Preparation of the media at the moment of use	410
	Melting of the agar in solid media	410
	Addition of supplements to basal media	410
	Distribution of solid media over plates	410
	Drying of media in plates intended for surface plating	410
	Deaeration of the media for anaerobic microorganisms	410
24.3	References	410
Annex 1. Preparation of media and reagents		411
	Revision history	411
	Acetamide agar/broth	412

Acetamide broth ISO	412
Acid phosphatase reagent	413
AE sporulation medium modified for <i>Clostridium perfringens</i>	413
Agar <i>Listeria</i> Ottaviani & Agosti (ALOA)	414
Agar plug (Agar 2%)	415
Agar plug with thioglycolate	415
Alcoholic solution of iodine	415
3:1 alcoholic solution of iodine	415
Alkaline peptone water (APW)	416
Alkaline saline peptone water (ASPW)	416
All purpose Tween (APT) agar/broth	416
All purpose Tween (APT) agar acidified	417
All purpose Tween (APT) agar sucrose BCP	417
All purpose Tween (APT) agar glucose	417
Ammonium iron (III) sulfate solution	417
Arginine glucose slants (AGS)	417
Asparagine broth	418
<i>Bacillus acidoterrestris</i> (BAT) agar/broth	418
Baird-Parker (BP) agar	418
Bile esculin agar	419
Bile esculin azide agar	419
Biosynth Chromogenic Medium (BCM®) <i>Listeria monocytogenes</i> (R&F <i>Listeria monocytogenes</i>)	420
Bismuth sulfite (BS) agar	420
Blood agar	421
Bolton Broth	421
Brain heart infusion (BHI) agar/broth	422
Brilliant green agar (BGA)	423
Brilliant green bile (BGB) broth	423
1% brilliant green solution	423
Brilliant green sulfa (BGS) agar	424
Brilliant green water	424
Bromcresol purple dextrose (BCP) broth	424
0.04% Bromothymol blue indicator	424
Buffered <i>Listeria</i> enrichment broth (BLEB)	425
Buffered peptone water (BPW)	425
Modifications	426
Buffered peptone water modified (mBPWp)	426
Butterfield's phosphate buffer (PB) (phosphate dilution water) (Butterfield's phosphate buffered dilution water)	426
Butterfield's phosphate buffer with 40% glucose	427
Carbohydrate fermentation medium	427
Cefsulodin irgasan novobiocin (CIN) agar	427
Cellobiose colistin (CC) agar	428
Cellulase solution	428
Cephalothin sodium fusidate ceftrimide (CFC) agar	429
Christensen urea agar	429
CHROMagar™ <i>Listeria</i>	430
CHROMagar™ <i>Vibrio</i>	430
Citrate azide agar	430
Chromogenic coliform agar (CCA)	431

Columbia Blood Agar (CBA)	431
Congo red magnesium oxalate (CR-MOX) agar	432
Cooked meat medium (CMM)	432
Coomassie brilliant blue solution	432
Chromogenic <i>Cronobacter</i> isolation (CCI) agar	433
<i>Cronobacter</i> selective broth (CSB)	433
Decarboxylase broth Falkow	434
Decarboxylation medium	434
Dextrose tryptone agar (DTA), dextrose tryptone broth (DTB)	435
DFI chromogenic agar	435
Dichloran 18% glycerol (DG18) agar	435
Dichloran rose Bengal chloramphenicol (DRBC) agar	436
Diluent with α -amylase	436
Dilution water (see magnesium chloride phosphate buffer, PBMgCl)	436
Dipotassium hydrogen phosphate solution (K_2HPO_4)	436
Dipotassium hydrogen phosphate (K_2HPO_4) solution with antifoam agent	437
Double modified lysine iron agar (DMLIA)	437
<i>E. coli</i> (EC) broth	438
<i>E. coli</i> broth with 4-methylumbelliferyl- β -D-glucuronide (EC-MUG)	438
Elliker agar/broth	438
<i>Enterobacteriaceae</i> enrichment broth (EEB)	439
m- <i>Enterococcus</i> agar (Slanetz & Bartley Medium)	439
Ethanol 70%	439
Fermentation medium for <i>Clostridium perfringens</i>	440
Ferric chloride solution 10%	440
Formalinized physiological saline solution	440
Fraser broth	441
β -Galactosidase reagent (ONPG reagent) (<i>o</i> -nitrophenyl- β -d-galactopyranoside)	441
Glucose agar	442
α -Glucosidase enzymatic assay solution	442
Glycerol salt solution buffered	443
Gram-stain reagents (Hucker)	443
Gum tragacanth and gum arabic mixture	444
Half Fraser broth (demi-Fraser broth)	444
Halotolerance saline peptone water	445
Hektoen enteric (HE) agar	445
Horse blood overlay medium (HL)	446
m-HPC agar	446
Hydrochloric acid solution	446
3% hydrogen peroxide (H_2O_2)	446
Indole Kovac's reagent (5% <i>p</i> -dimethylaminobenzaldehyde solution)	447
Indoxyl acetate discs (2.5 to 5.0 mg)	447
Irgasan ticarcillin potassium chlorate (ITC) broth	447
Iron milk medium modified	448
K agar	448
KF <i>Streptococcus</i> agar	449
KF <i>Streptococcus</i> broth	449
Kim-Goepfert (KG) agar	449
King's B medium	450
Koser's citrate broth	450

Lactose broth (LB)	451
Lactose broth supplemented with anionic Tergitol 7 or Triton X-100	451
Lactose broth supplemented with cellulase solution	451
Lactose broth supplemented with papain solution	451
Lactose gelatin medium	451
Lactose sulfite (LS) medium (LS)	452
Lauryl sulfate tryptose (LST) broth	452
Levine's eosin-methylene blue (L-EMB) agar	453
Liver broth	453
Liver veal agar (LVA)	454
Lysine iron agar (LIA)	454
MacConkey (MAC) agar	454
MacFarland standards	455
Magnesium chloride phosphate (PBMgCl) buffer	455
Malonate broth	455
Malt acetic agar (MAA)	456
Malt extract agar (MEA) with antibiotics	456
Malt extract broth (MEB)	457
Malt extract yeast extract 40% glucose (MY40G)	457
Mannitol egg yolk polymyxin (MYP) agar	457
de Man Rogosa & Sharpe (MRS) agar/broth	458
<i>m-Enterococcus</i> agar (Slanetz & Bartley Medium)	458
Methyl red solution	459
Modified cellobiose polymyxin colistin (mCPC) agar	459
Modified charcoal cefoperazone deoxycholate (mCCDA) agar	460
Modified Oxford (MOX) agar	460
Modified semisolid Rappaport-Vassiliadis (MSRV) agar	461
Modified University of Vermont (UVM) broth	462
Morpholinepropanesulfonic acid-buffered <i>Listeria</i> enrichment broth (MOPS-BLEB)	463
Motility medium for <i>Bacillus cereus</i>	463
Motility nitrate medium	463
Motility test medium	464
Motility test medium ISO	464
MR-VP broth	465
Muller-Kauffmann tetrathionate novobiocin (MKTTn) broth	465
Nessler reagent	466
Ninhydrin solution (3.5% mass/volume)	466
Nitrate broth	466
Nitrate test reagents	467
Nitrate test reagents ISO 7937:2004	467
Nutrient agar (NA), nutrient broth (NB)	468
Nutrient agar with manganese (NAMn)	468
Nutrient agar with trypan blue	468
Nutrient broth with lysozyme	468
NWRI agar (HPCA)	468
Orange serum agar (OSA), orange serum broth (OSB)	468
Oxford agar (OXA)	469
Oxidase Kovac's reagent (1% <i>N,N,N,N</i> -tetramethyl- <i>p</i> -phenylenediamine dihydrochloride aqueous solution)	470
Oxidation fermentation (OF) glucose agar	470

Oxoid Chromogenic <i>Listeria</i> Agar (OCLA)	470
Papain solution 5%	471
PE-2 medium	471
Penicillin pimaricin agar (PPA)	471
Peptone sorbitol bile (PSB) broth	472
Peptone water (PW)	472
Phenol red carbohydrate broth	472
Phenylalanine (tryptophane) deaminase agar	473
Phosphate buffered saline (PBS)	473
Phosphate buffered solution according to ISO 6887-4:2017	474
Phosphate buffered solution according to ISO 6887-5:2010	474
Phosphate saline buffer 0.02M (pH 7.3 to 7.4)	474
Plate count agar (PCA) standard methods agar (SMA), (tryptone glucose yeast extract agar)	475
Plate count agar (PCA) supplemented with 0.1% soluble starch	475
Plate count agar (PCA) with chloramphenicol (100 mg/L)	475
Potassium cyanide broth (KCN)	475
0.5% Potassium hydroxide saline solution	476
Potato dextrose agar (PDA) acidified	476
Potato dextrose agar (PDA) with antibiotics	477
Preservative-resistant yeast (PRY) medium	477
Preston broth	478
<i>Pseudomonas</i> CN agar	478
Purple agar/broth for carbohydrate fermentation	479
Pyrazinamidase agar	479
R2A agar	480
Rapid'L.mono agar	480
Rappaport-Vassiliadis (R-10) broth	481
Rappaport-Vassiliadis (RV) medium	481
Rappaport-Vassiliadis soya (RVS) broth	482
Reconstituted nonfat dry milk	483
Reinforced clostridial medium (RCM)	483
Reinforced clostridial medium (RCM) with lactate	484
R&F <i>Cronobacter</i> Chromogenic Agar	484
R&F <i>Listeria monocytogenes</i> (see Biosynth Chromogenic Medium (BCM®) <i>Listeria monocytogenes</i>)	484
Ringer's solution quarter strength	484
Rogosa SL agar/broth	485
Saline decarboxylase broth	485
Saline nutrient agar (SNA)	486
Saline peptone water (SPW) (peptone salt solution)	486
Saline tryptophan broth	486
Selenite cystine (SC) broth	487
Sheep blood agar	488
Simmons citrate agar	488
Sodium citrate solution ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)	489
0.5% Sodium desoxycholate solution	489
Sodium dodecyl sulfate polymixin sucrose (SDS) agar	489
Sodium hippurate solution	490
Sodium hydroxide solutions	490
Sodium tripolyphosphate solution	490
Spore stain reagents (Ashby's)	490

Sudan black B solution 0.3% in ethanol 70%	491
Sulfide indole motility (SIM) medium	491
Sulfite agar	491
T ₁ N ₁ agar and T ₁ N ₁ broth	492
T ₁ N ₀ and T ₁ N ₃ broth	492
Tetrathionate (TT) broth	492
Tetrathionate broth Hajna and Damon (1956) (TTH)	493
Thermoacidurans agar (TAA) and thermoacidurans broth (TAB)	494
Thioglycollate medium (TGM) fluid	494
Thiosulfate citrate bile sucrose (TCBS) agar	494
Toluidine blue DNA agar	495
Triphenyltetrazolium chloride soya tryptone (TSAT) agar	495
Triple sugar iron (TSI) agar	496
Trypticase soy agar/broth (TSA/TSB)	496
Trypticase soy agar/broth with 0.6% yeast extract (TSA-YE or TSB-YE)	497
Trypticase soy broth (TSB) with 10% NaCl and 1% sodium pyruvate	497
Trypticase soy broth (TSB) with 20% NaCl	497
Trypticase soy broth (TSB) with polymyxin	497
Trypticase soy broth (TSB) with 0.5% potassium sulfite (K ₂ SO ₃)	497
Trypticase soy broth (TSB) with 35 mg/L ferrous sulfate	497
Trypticase soy agar (TSA) with 5% sheep blood	497
Tryptone glucose extract (TGE) agar	497
Tryptone glucose yeast extract acetic (TGYA) agar	498
Tryptone glucose yeast extract acetic broth (TGYAB)	498
Tryptone (tryptophan) broth	498
Tryptose sulfite cycloserine (TSC) agar	499
Tween esterase test medium	500
Tyrosine agar	500
Universal pre-enrichment broth	500
Urea agar (Christensen)	501
Urea broth	501
Urea broth rapid	502
Vaspar	502
Violet red bile (VRB) agar	502
Violet red bile glucose (VRBG) agar	502
Voges-Proskauer (VP) broth modified for <i>Bacillus</i>	503
Voges-Proskauer (VP) test reagents (5% α-naphthol alcoholic solution, 40% potassium hydroxide aqueous solution)	503
Xylose lysine desoxycholate agar (XLD)	504
Xylose lysine Tergitol 4 (XLT4) agar	504
Yeast extract starch glucose (YSG) agar/broth	504
Annex 2. Sampling plans and microbiological limits recommended by ICMSF for foods	507
Index	515

About the authors



Neusely da Silva is a scientific researcher at the Food Technology Institute (ITAL), a government research agency of the State of São Paulo, Brazil. She graduated in Food Engineering and has a PhD in Food Science from the State University of Campinas (UNICAMP, Brazil). Director of the Microbiology Reference Laboratory of the Food Technology Institute from 1995 to 2007, she was responsible for the accreditation of the laboratory assays according to ISO 17025. She is author of over 70 publications in the area of Food Microbiology and her major research areas are bacterial physiology and methods for detection of bacteria responsible for food-borne diseases and bacteria responsible for food spoilage. E-mail: neusely@ital.sp.gov.br.



Marta Hiromi Taniwaki, PhD, is a scientific researcher at the Food Technology Institute (ITAL) at the Center of Quality and Food Science in Campinas, Brazil. She graduated in Biology and has a PhD in Food Science and Technology from the University of New South Wales, Australia. She is author of over 100 publications in the area of Food Mycology, Mycotoxins and Food Microbiology. She is member of the International Commission on Food Mycology (ICFM) since 1997; member of the Brazilian delegation at the Codex Contaminants in Food (CCCF) since 2006; Member of the International Commission on Microbiological Specifications for Foods (ICMSF) since 2010 and editorial board of Mycotoxin Research since 2012. Her major research areas are: fungi and mycotoxins in foods; biodiversity of toxigenic fungi in foods, fungal physiology and mycotoxin production, polyphasic approach to biosystematics of *Aspergillus* species. E-mail: marta@ital.sp.gov.br



Valéria Christina Amstalden Junqueira was a scientific researcher at the Food Technology Institute (ITAL) at the Center of Quality and Food Science (CCQA) in Campinas, SP, Brazil from 1988 to 2016 and Director of the Microbiology Reference Laboratory at ITAL from 2011 to 2015. She graduated in Biology and has PhD in Food Technology from the State University of Campinas (UNICAMP, Brazil) in the area of hygiene and legislation of foods. She has been a member of the Brazilian delegation at the Codex Committee on Food Hygiene (CCFH) since 2002. Her major research activities are on the control of the microbiological quality of food with an emphasis on anaerobic bacteria, spoilage microorganisms of processed foods, microbiological quality of water and non-alcoholic beverages. E-mail: valeriaca@gmail.com.



Neliane Ferraz de Arruda Silveira is a scientific researcher at the Food Technology Institute (ITAL) at the Center of Quality and Food Science in Campinas, Brazil. She is a biologist with PhD in Food Technology from the State University of Campinas (UNICAMP, Brazil), in the area of hygiene and legislation of foods. Her major research areas are the control of microbiological quality of food with emphasis on fish and fish products, minimally processed vegetables, foods served in collective meals, meat products and bacteriological quality of drinking water. E-mail: neliane@ital.sp.gov.br



Margarete Midori Okazaki is a scientific researcher at the Food Technology Institute (ITAL), at the Center of Quality and Food Science in Campinas, Brazil. She graduated in Food Engineering and has a Master Science degree (Msc.) in Food Technology from the State University of Campinas (UNICAMP, Brazil).

She is vice director of the Microbiology Reference Laboratory of the Food Technology Institute and concentrates her activities on the control of the microbiological quality of food and water and on technical training focused on microbiological examination methods. E-mail: okazaki@ital.sp.gov.br



Renato Abeilar Romeiro Gomes is a scientific researcher at the Food Technology Institute (ITAL), Campinas, Brazil. He graduated in Agricultural Engineer and has a Master's degree in Agricultural Engineering from the Federal University of Viçosa, with MBA specialization. He is currently a researcher at the Dairy Technology Center of the Food Technology Institute. E-mail: rarg@ital.sp.gov.br.

Preface

This manual was prepared with standardized methods published by renowned international organizations such as International Organization for Standardization (ISO), American Public Health Association (APHA), AOAC International, Food and Drug Administration (FDA) and United States Department of Agriculture (USDA).

The manual includes methods for the enumeration of indicator microorganisms of general contamination (total aerobic mesophilic bacteria, lactic acid bacteria, yeasts and molds), indicators of hygiene and sanitary conditions (coliforms, *E. coli*, enterococci), spore-forming bacteria (aerobic thermophilic and mesophilic bacteria, anaerobic thermophilic and mesophilic bacteria, *Alicyclobacillus*), spoilage fungi (thermoresistant molds, osmophilic yeasts, preservative resistant yeasts) and pathogenic bacteria (*Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Cronobacter*, *Campylobacter*, *Yersinia enterocolitica*, *Vibrio cholerae*, *Vibrio parahaemolyticus*). The chapter covering the examination of water includes methods for the detection and determination of coliforms, *E. coli*, *Pseudomonas aeruginosa*, *Clostridium perfringens* and enterococci.

The major objective of the book is to provide an illustrated laboratory manual with an overview of current standard microbiological methods for the examination of food and water. The didactic setup and the visualization of procedures in step-by-step schemes allows student and practitioner to quickly perceive and execute the procedure intended. Each chapter provides numerous methods for a certain examination, and also provides simple or quick alternatives. The chapters' introductions summarize the existing knowledge regarding the target microorganism(s) and present the most useful information available in literature.

The book is intended for laboratory education of undergraduate and graduate students in food engineering and related disciplines and as an up-to-date practical companion for researchers, analysts, technicians and teachers.

In this second edition several methods have been revised according to new editions of the references used, including:

- The *Compendium of Methods for the Microbiological Examination of Foods*, 5th edition (2015), from American Public Health Association (APHA).
- The *Standard Methods for the Examination of Water & Wastewater*, 22nd edition (2012), from American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF).
- The *Official Methods of Analysis of AOAC International*, 20th edition (2016), from AOAC International.
- The *Bacteriological Analytical Manual* [BAM Online] from Food and Drug Administration (last update).
- The *Microbiology Laboratory Guidebook* [MLG Online], from Food Safety and Inspection Service, United States Department of Agriculture (last update).

New methods have also been described, including:

- Aerobic mesophilic bacteria in foods – plate count methods ISO 4833-1/2:2013
- Method ISO 21528-1/2:2017 for *Enterobacteriaceae* in Foods
- Method BAM/FDA:2017 for Coliforms and *E. coli* in Food
- Method ISO 9308-1:2014 for Coliforms and *E. coli* in Water
- Method BAM/FDA:2012 for *Cronobacter* in Infant Formula
- *Clostridium perfringens* in water – membrane filtration method ISO 14189:2013
- *Clostridium perfringens* in foods – plate count method ISO 7937:2004
- Lactic acid bacteria in foods - Plate count method ISO 15214:1998



Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

Tables

Table 2.1	Diluents recommended by different regulatory agencies for food and water analysis.	15
Table 3.1	Examples for calculating the pour plate results in not ideal conditions.	34
Table 3.2	Weighted means and confidence intervals for relevant numbers of colonies according to ISO 14461-2:2005.	37
Table 4.1	Guide for the use of the MPN tables.	51
Table 4.2	Examples for use the MPN tables.	52
Table MPN-1	Most probable number (MPN) and 95% confidence intervals for three tubes each at 0.1, 0.01, and 0.001 g or mL inocula.	54
Table MPN-2	Most probable number (MPN) and 95% confidence intervals for five tubes each at 0.1, 0.01, and 0.001 g or mL inocula.	55
Table MPN-3	Most probable number (MPN) and 95% confidence intervals for 10 tubes at 10 mL inocula.	56
Table MPN-4	Most probable number (MPN) and 95% confidence intervals for five tubes at 20 mL inocula.	56
Table MPN-5	Most probable number (MPN) and 95% confidence intervals for five tubes at 10 mL inocula.	56
Table 6.1	Typical mesophilic aerobic plate counts of selected foods.	66
Table 6.2	FAO/WHO microbiological specifications for foods.	66
Table 6.3	APHA standards for mesophilic aerobic plate count in milk and dairy products.	66
Table 6.4	Media and incubation conditions recommended by APHA, FDA and ISO methods for plate count of aerobic mesophilic and psychrotrophic bacteria in foods and water.	67
Table 6.5	Analytical kits adopted as AOAC Official Methods for mesophilic aerobic plate count in foods.	68
Table 7.1	Media and incubation conditions recommended by APHA, FDA and ISO methods for analysis of yeasts and molds in foods.	83
Table 7.2	Analytical kits adopted as AOAC Official Methods for the yeasts and molds count in foods.	83
Table 7.3	Tolerance of heat-resistant molds isolated from foods and beverages.	84
Table 8.1	Media and incubation conditions recommended by APHA, AOAC and ISO methods for <i>Enterobacteriaceae</i> in foods.	104
Table 9.1	Media and incubation conditions recommended by APHA and ISO methods for total coliforms, thermotolerant coliforms and <i>E. coli</i> in foods and water.	118
Table 9.2	Analytical kits adopted as AOAC Official Methods for coliforms and <i>E. coli</i> in foods.	120
Table 10.1	Biochemical and growth characteristics of the species and subspecies of coagulase positive <i>Staphylococcus</i> .	136
Table 10.2	Analytical kits adopted as AOAC Official Methods for <i>Staphylococcus aureus</i> in foods.	139
Table 11.1	Differential characteristics of the species of <i>Bacillus cereus</i> group.	150
Table 12.1	Classification of <i>C. perfringens</i> into types based on the production of the alpha, beta, epsilon, and iota toxins.	162
Table 12.2	Media and incubation conditions recommended by APHA, FDA and ISO methods for sulfite reducing clostridia and <i>Clostridium perfringens</i> in foods and water.	163
Table 13.1	Characteristics most used to differentiate <i>Enterococcus</i> from group bovis <i>Streptococcus</i> .	180
Table 13.2	Media and incubation conditions recommended by APHA, APHA/AWWA/WEF and ISO methods for enterococci and fecal streptococci in foods and water.	181

Table 14.1	Main characteristics of the lactic acid bacteria associated with foods.	190
Table 14.2	Culture media for lactic acid bacteria counts in foods, their main applications and forms of use.	197
Table 15.1	Biochemical and growth characteristics of the species and subspecies of <i>Campylobacter</i> described by 2 nd edition of <i>Bergey's Manual of Systematic Bacteriology</i> .	208
Table 15.2	Characteristics of <i>Campylobacter</i> species growing at 41.5°C.	213
Table 16.1	Biochemical characteristics used to differentiate the new genus <i>Cronobacter</i> from some other genera of the <i>Enterobacteriaceae</i> family.	218
Table 16.2	Biochemical tests used to differentiate the species and subspecies of the genus <i>Cronobacter</i> .	219
Table 16.3	Microbiological criteria applied by the Codex Alimentarius to powdered infant formulae (finished product).	220
Table 16.4	Media and incubation conditions recommended by ISO and FDA methods for <i>Cronobacter</i> in foods.	221
Table 16.5	Guide for the interpretation of <i>Cronobacter</i> spp. confirmatory tests according the method ISO 22964:2017.	223
Table 17.1	Changes in the nomenclature of members of the genus <i>Pseudomonas</i> .	228
Table 17.2	Characteristics differentiating the strains of Group III <i>Shewanella putrefaciens</i> "senso stricto," <i>Shewanella algae</i> , <i>Shewanella baltica</i> and <i>Shewanella putrefaciens</i> .	231
Table 17.3	Guide for the interpretation of <i>Pseudomonas aeruginosa</i> confirmatory tests according the method ISO 16266:2006.	236
Table 18.1	Characteristics for differentiating the six original species of the genus <i>Listeria</i> described until the publication of the 2 nd Edition of <i>Bergey's Manual of Systematic Bacteriology</i> .	245
Table 18.2	Characteristics used by Weller <i>et al.</i> (2015) for differentiating <i>Listeria</i> species, including those described after the publication of the 2 nd Edition of <i>Bergey's Manual of Systematic Bacteriology</i> .	245
Table 18.3	Media and incubation conditions recommended by FDA, USDA and ISO methods for <i>Listeria monocytogenes</i> in foods.	247
Table 18.4	Analytical kits adopted as AOAC Official Methods for <i>Listeria monocytogenes</i> in foods.	249
Table 18.5	Guide for the interpretation of <i>Listeria monocytogenes</i> and <i>Listeria</i> spp. confirmatory tests according the method ISO 11290-2:2017.	260
Table 19.1	The two systems of validly published names of <i>Salmonella</i> .	267
Table 19.2	Biochemical reactions of <i>Salmonella</i> species, subspecies and serovars important epidemiologically.	271
Table 19.3	Media and incubation conditions recommended by ISO, FDA and USDA methods for <i>Salmonella</i> in foods.	273
Table 19.4	Analytical kits adopted as AOAC Official Methods for <i>Salmonella</i> in foods.	275
Table 19.5.A	Guide for the interpretation of <i>Salmonella</i> confirmatory biochemical tests according the method ISO 6579-1:2017.	281
Table 19.5.B	Guide for the interpretation of <i>Salmonella</i> confirmatory serological tests according the method ISO 6579-1:2017.	281
Table 19.6	Guide for selecting pre-enrichment broths, dilution ratios and eventual variations in the pre-enrichment procedure for <i>Salmonella</i> analysis using the BAM/FDA method.	284
Table 19.7	Guide for selecting TSI and LIA cultures for confirmation tests according the method BAM/FDA 2011.	288
Table 19.8	Guidance for sample preparation and pre-enrichment of <i>Salmonella</i> in method MLG/USDA:2017.	294
Table 19.9	Guide for selecting TSI and LIA cultures for confirmation tests according the method MLG/USDA:2018.	295
Table 20.1	Key characteristics used to differentiate the pathogenic <i>Vibrio</i> in groups.	301
Table 20.2	Biochemical characteristics of pathogenic <i>Vibrio</i> .	302

Table 20.3	Characteristics used to differentiate <i>V. cholerae</i> O1 biotypes classic and El-Tor.	304
Table 20.4	Characteristics of the colonies of some species of <i>Vibrio</i> on CHROMagar and TCBS.	306
Table 20.5	Guide for the interpretation of presumptive potentially enteropathogenic <i>V. cholerae</i> or <i>V. parahaemolyticus</i> confirmatory tests according to the method ISO 21872-1:2017.	322
Table 21.1	Differential characteristics of the species of the genus <i>Yersinia</i> .	326
Table 21.2	Differentiation of biogroups of <i>Yersinia enterocolitica</i> .	327
Table 21.3	The biovars of <i>Yersinia enterocolitica</i> according to the ISO 10273:2017.	332
Table 22.1	Guide for the interpretation of <i>Alicyclobacillus</i> confirmatory tests according to the method IFU 12:2007.	367
Table 23.1	D and z values of several microorganisms of importance in foods.	377
Table 23.2	Keys to probable cause of spoilage in low acid canned foods.	387
Table 23.3	Keys to probable cause of spoilage in acid or acidified canned foods.	395
Table 24.1	Condition recommended in the 22 nd Edition of the <i>Standard Methods for the Examination of Water and Wastewater</i> (Hunt, 2012) for storage of culture media (part 9020B, Table 9020:V).	409



Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

Figures

Figure 2.1	Points recommended by ISO 17604:2015 for swab sampling of bovine carcasses.	12
Figure 2.2	Points recommended ISO 17604:2015 for swab sampling of swine carcasses.	12
Figure 5.1	Streak plating technique for obtaining pure cultures.	63
Figure 6.1	Scheme of analysis for enumeration of aerobic mesophilic bacteria in foods using the plate count method APHA 8:2015.	70
Figure 6.2	Scheme of analysis for enumeration of aerobic psychrotrophic bacteria in foods using the plate count method APHA 13.61:2015.	74
Figure 6.3	Scheme of analysis for the enumeration of aerobic mesophilic bacteria in foods using the plate count method ISO 4833-1:2013 or ISO 4833-2:2013/Corr.1:2014.	76
Figure 6.4	Scheme of analysis for enumeration of aerobic mesophilic bacteria in frozen, chilled, precooked or prepared foods using the plate count method BAM/FDA:2001.	77
Figure 7.1A	Scheme of analysis for enumeration of yeasts and molds in foods using the plate count method APHA 21:2015.	88
Figure 7.1B	Scheme of analysis for enumeration of yeasts and molds in foods using the plate count methods ISO 21527-1:2008 and ISO 21527-2:2008.	91
Figure 7.2	Scheme of analysis for enumeration of psychrotrophic fungi in foods using the plate count method APHA 13:2015.	94
Figure 7.3	Scheme of analysis for enumeration of heat-resistant molds in foods using the plate count method APHA 22.4:2015.	96
Figure 7.4A	Scheme of analysis for determination of preservative-resistant yeasts in foods using the presence/absence method described by Pitt and Hocking (2009).	97
Figure 7.4B	Scheme of analysis for determination of preservative-resistant yeasts in foods using the plate count method described by Pitt and Hocking (2009).	98
Figure 7.5	Scheme of analysis for the enumeration of osmophilic yeasts in foods using the membrane filtration or the plate count method APHA 17.3:2015.	100
Figure 8.1	Scheme of analysis for the enumeration of <i>Enterobacteriaceae</i> in foods using the plate count method APHA 9.62:2015.	105
Figure 8.2	Scheme of analysis for the enumeration of <i>Enterobacteriaceae</i> in foods using the MPN method APHA 9.61:2015.	107
Figure 8.3	Scheme of analysis for the enumeration of <i>Enterobacteriaceae</i> in foods using the plate count method ISO 21528-2:2017.	110
Figure 8.4	Scheme of analysis for the enumeration of <i>Enterobacteriaceae</i> in foods using the P/A or MPN method ISO 21528-1:2017.	112
Figure 9.1	Scheme of analysis for the enumeration of total and thermotolerant coliforms and <i>E. coli</i> in foods using the MPN method APHA 9:2015.	121
Figure 9.2	Scheme of analysis for the enumeration of total coliforms and presumptive <i>E. coli</i> in foods using the most probable number (MPN) methods ISO 4831:2006 and ISO 7251:2005.	124
Figure 9.3	Scheme of analysis for the enumeration total and thermotolerant coliforms and <i>E. coli</i> in water using the most probable number (MPN) method APHA/AWWA/WEF:2012.	127
Figure 9.4	Scheme of analysis for the enumeration of total coliforms in foods using the plate count method APHA:2015.	131
Figure 9.5	Scheme of analysis for the enumeration of coliforms and <i>E. coli</i> in water using the membrane filtration method ISO 9308-1:2014/Amd.1:2016.	133

Figure 10.1	Scheme of analysis for the enumeration of coagulase positive staphylococci and <i>Staphylococcus aureus</i> in foods using the plate count method APHA 39.63:2015.	141
Figure 10.2	Scheme of analysis for the enumeration of coagulase positive staphylococci and <i>Staphylococcus aureus</i> in foods using the most probable number (MPN) method APHA 39.62:2015.	144
Figure 10.3	Scheme of analysis for the detection of coagulase positive staphylococci and <i>Staphylococcus aureus</i> in foods using the presence/absence method APHA 29.61:2015.	146
Figure 11.1	Scheme of analysis for the enumeration of <i>Bacillus cereus</i> in foods using the plate count method APHA 31.61:2015.	155
Figure 11.2	Scheme of analysis for the enumeration of <i>Bacillus cereus</i> in foods using the Most Probable Number (MPN) method APHA 31.62:2015.	158
Figure 12.1	Scheme of analysis for the enumeration of <i>Clostridium perfringens</i> in foods using the plate count method APHA 33.72:2015.	166
Figure 12.2	Scheme of analysis for the detection of <i>Clostridium perfringens</i> in foods using the presence/absence method APHA 33.71:2015.	168
Figure 12.3	Scheme of analysis for the enumeration of <i>Clostridium perfringens</i> in water using the membrane filtration method ISO 14189:2013.	174
Figure 13.1	Scheme of analysis for the enumeration of enterococci and fecal streptococci in foods using the plate count method APHA 10.5:2015.	182
Figure 13.2	Scheme of analysis for the enumeration of enterococci and fecal streptococci using the most probable number (MPN) method APHA 10.2:2015.	183
Figure 13.3	Scheme of analysis for the enumeration of enterococci and fecal streptococci using the membrane filtration method APHA/AWWA/WEF 9230C.3.c:2012.	185
Figure 13.4	Scheme of analysis for the enumeration of intestinal enterococci in water using the membrane filtration method ISO 7899-2:2000.	186
Figure 14.1	Scheme of analysis for the enumeration of lactic acid bacteria in foods using the plate count method APHA 19.52:2015.	199
Figure 14.2	Scheme of analysis for the enumeration of heterofermentative lactic acid bacteria in foods using the most probable number (MPN) method APHA 19.526:2015 with MRS broth.	201
Figure 14.3	Scheme of analysis for the enumeration of lactic acid bacteria in foods using the most probable number (MPN) method APHA 19.524:2015 with Rogosa SL broth.	203
Figure 14.4	Scheme of analysis for enumeration of lactic acid bacteria in foods using the plate count methods ISO 15214:1998.	204
Figure 15.1	Scheme of analysis for detection of thermotolerant <i>Campylobacter</i> in foods using the presence/absence method ISO 10272-1:2017.	211
Figure 15.2	Scheme of analysis for detection of thermotolerant <i>Campylobacter</i> in foods using the plate count method ISO 10272-2:2017.	215
Figure 16.1	Scheme of analysis for detection of <i>Cronobacter</i> in foods using the presence/absence method ISO 22964:2017.	222
Figure 16.2	Scheme of analysis for detection of <i>Cronobacter</i> in dehydrated powdered infant formula using the presence/absence method BAM/FDA:2012.	224
Figure 17.1	Scheme of analysis for the enumeration of <i>Pseudomonas aeruginosa</i> in water using the Most Probable Number (MPN) method APHA/AWWA/WEF 9213:2012.	233
Figure 17.2	Scheme of analysis for the enumeration of <i>Pseudomonas aeruginosa</i> in water using the membrane filtration method ISO 16266:2006.	235
Figure 17.3	Scheme of analysis for the enumeration of presumptive <i>Pseudomonas</i> spp. in meat and meat products using the plate count method ISO 13720:2010.	238
Figure 17.4	Scheme of analysis for the enumeration of <i>Pseudomonas</i> spp. in milk and milk products using the plate count method ISO 11059:2009.	239

Figure 18.1	Scheme of analysis for detection of <i>Listeria monocytogenes</i> in foods using the presence/absence method BAM/FDA:2017.	251
Figure 18.2	Scheme of analysis for detection of <i>Listeria monocytogenes</i> in foods using the presence/absence method USDA/MLG:2017.	255
Figure 18.3	Scheme of analysis for detection of <i>Listeria monocytogenes</i> in foods using the presence/absence method ISO 11290-1:2017.	261
Figure 19.1	Scheme of analysis for detection of <i>Salmonella</i> in foods using the presence/absence method ISO 6579-1:2017.	278
Figure 19.2	Scheme of analysis for detection of <i>Salmonella</i> in foods using the presence/absence method BAM/FDA:2018.	283
Figure 19.3	Scheme of analysis for detection of <i>Salmonella</i> in foods using the presence/absence method MLG/USDA:2017.	293
Figure 20.1A	Scheme of analysis for detection of <i>Vibrio cholerae</i> in foods using the presence/absence method BAM/FDA 2004.	308
Figure 20.1B	Scheme of analysis for detection of <i>Vibrio cholerae</i> in foods and water using the presence/absence method APHA 40.61:2015.	311
Figure 20.2A	Scheme of analysis for the enumeration of <i>Vibrio parahaemolyticus</i> in foods using the most probable number (MPN) method BAM/FDA 2004.	314
Figure 20.2B	Scheme of analysis for the enumeration of <i>Vibrio parahaemolyticus</i> in foods using the presence/absence method APHA 40.62/40.63:2015.	317
Figure 20.3	Scheme of analysis for detection of presumptive enteropathogenic <i>Vibrio cholerae</i> and <i>Vibrio parahaemolyticus</i> in foods using the presence/absence method ISO 21872-1:2017.	319
Figure 22.1	Scheme of analysis for the enumeration of spores of total and “flat sour” thermophilic aerobic sporeformers in foods using the methods APHA 26:2015.	355
Figure 22.2	Scheme of analysis for the enumeration of mesophilic aerobic sporeformers in foods using the methods APHA 23:2015.	360
Figure 23.1	Survival curve and determination of the D value.	375
Figure 23.2	Thermal destruction curve and determination of the z value.	376
Figure 23.3	Scheme of analysis for testing commercial sterility or cause of spoilage of low-acid canned foods using the method APHA:2015.	383
Figure 23.4	Sanitary (bacteriological) can opener for opening cans in the commercial sterility test.	384
Figure 23.5	Scheme of analysis for testing commercial sterility or cause of spoilage of acid canned foods using the method APHA:2015.	391



Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

1 Sampling, transport and storage of samples for analysis

Revision history

Item 1.2.1.c (revised) Sterilization of flasks and utensils should be done at $121\pm 3^\circ\text{C}$ for at least 15 minutes in autoclave. In sterilization oven should be made at $170\pm 10^\circ\text{C}$ for at least one hour (ISO 7218:2007/Amd.1:2013).

Item 1.2.4 (revised) Suppressed in the 22nd edition *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) the recommendation to add EDTA to water samples with high metal content.

Item 1.3.5 (revised) The storage temperature of water samples under refrigeration recommended by the 22nd edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) changed from 10°C to 8°C , emphasizing the recommendation that these samples should not be frozen.

1.1 Introduction

Most of the recommendations and guidelines contained in this chapter are taken from the American Public Health Association (APHA), as described in the 5th Edition of the *Compendium of Methods for Microbiological Examination of Foods* (Salfinger and Tortorello, 2015). When different from or complementary to those of the *Compendium*, they were complemented with information and recommendations from the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012), specific to the analysis of water, the 17th Edition of the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004), specific to the microbiological examination of dairy products and the standards of the International Organization for Standardization (ISO 6887-4:2017; ISO 7218:2007/Amd.1:2013), recommended for performing tests using ISO methods.

Some of the terms used throughout this text come from the terminology used by the International Commission on Microbiological Specification for Foods (ICMSE, 1986) for lot sampling, and their meaning should be thoroughly understood.

1.1.1 Lot

A lot is defined as an amount of food of the same composition and physical, chemical and sensory characteristics, produced and handled in one and the same production run and under exactly the same processing conditions. In practice, a lot generally is the quantity of food produced within a certain time interval during an uninterrupted period of processing of a production line.

1.1.2 Lot sample and sample unit

A lot sample is a fraction of the total amount produced, withdrawn randomly, to evaluate the conditions of the lot. In the case of foods filled into individual packages, a lot sample is composed of n individual packages. In the case of bulk foods, which are not filled into individual packages, a lot sample is composed of n aliquots of a measured volume or weight of the product. Individual packages or aliquots are called sample units and – for the purpose of assessing the lot – are examined individually. From the combined results of analysis relative to n sample units, it is possible to infer the characteristics

of the lot as a whole, although the result of the examination of one single sample unit may never be taken as representative of the lot.

In *Salmonella* tests the criterion for foods is absence in any of the sample units examined. In such a case it is common to composite (mix together) sample units to perform one single analysis. The presence of *Salmonella* in the composite sample is unacceptable, irrespective of how many or which sample units are contaminated. Greater details will be presented in the specific chapter on *Salmonella*.

1.1.3 Lot sampling plans

Whenever the goal is to evaluate lots or batches, the taking of n sample units must follow a statistically adequate sampling plan. The most commonly used are the two- or three-class plans established by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002, 2011).

The two-class sampling plan classifies lots into two categories, acceptable or unacceptable, depending on the analysis results of n sample units. Two-class sampling plans are used more in the case of presence/absence tests, such as *Salmonella*, for example, in which absence is acceptable and the presence in any of the n sample units is unacceptable.

The three-class sampling plan classifies the lots into one of three categories: (1) acceptable; (2) intermediate quality but marginally acceptable; and (3) unacceptable. Three-class sampling plans are recommended for quantitative tests, for which the goal or standard is not the absence, but values that fall within a range between m and M . The parameters used in these plans for making decisions regarding the lots tested are:

n : the number of sample units that need to be randomly taken from one and the same lot and which are to be examined individually. The n sample units constitute the representative sample of the lot. As for non-quantitative and presence/absence tests (*Salmonella* or *Listeria monocytogenes*, for example) the sample units may be composite or pooled and subjected to a single analysis. However, when pooling or compositing samples the instructions and guidelines described in the chapters dealing with the specific tests in question should be consulted and strictly adhered to.

m : the microbiological-limiting criterion established for a given microorganism, in a given food. In a three-class sampling plan, this value separates an acceptable

lot from a lot of intermediate but marginally acceptable quality.

M : a tolerable limit, above the microbiological limiting criterion m , and which may be reached by a certain number (c) of sample units, but may not be exceeded by any of these. In a two-class plan, M separates an acceptable lot from an unacceptable one. In a three-class plan, M separates a lot of intermediate but marginally acceptable quality from an unacceptable lot.

c : among the n sample units that constitute a representative sample of the lot, c is the maximum number of units that may be accepted with counts above the limiting criterion m , provided none of these units exceed the M limit. In those cases in which the microbiological criterion is absence, c is equal to zero, and consequently, the two-class sampling plan is to be applied.

1.1.4 Analytical unit

A sample unit generally contains a quantity of product greater than necessary for performing the analysis, for the simple reason that, when collecting a sample unit, it is important to collect quantities enough to allow for storing counter-samples and preventing accidental losses. The analytical unit is the amount of food that is actually used to perform one or more tests on the sample unit. The number of analytical units necessary for the analysis depends on the number and types of tests that will be performed on one and the same sample unit; that is, one for general quantification tests (total aerobic mesophilic counts, yeast and mold counts, total coliform/fecal coliform/*Escherichia coli* counts, *Staphylococcus aureus* counts, *Bacillus cereus* counts, *Clostridium perfringens* counts), one for each presence/absence test (*Salmonella*, *Listeria monocytogenes* and all the tests requiring enrichment in specific broth) and one for any other test that requires the sample to be subjected to a differentiated treatment (counts of spore-forming bacteria, counts of heat-resistant molds and others).

1.2 Collecting samples for analysis

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that, whenever possible, samples packaged in individual packages should be collected and sent to the laboratory in their original commercial packaging, sealed and intact. Each packaging unit of an individual package of the product constitutes a sample unit and

as many sample units should be collected as required by the sampling plan. If the packaging unit or individual package contains an amount of food insufficient for performing the required analyses and the keeping of counter-samples, a sufficient number of individual packages should be collected as part of one and the same sample unit. At the time of analysis, the contents of these individual packages should be placed together in one single sterile flask, which must be subsequently thoroughly mixed before withdrawing an analytical unit of the mixture. If the product does not allow for such mixing, the analyst should take, from each of the individual packages, portions of approximately equal weight, to compose or complete the analytical unit for that particular sample unit.

In the case of foods contained in vats, tanks or large containers, impossible to be transported to the laboratory, representative portions should be transferred from the bulk product to sterile collecting flasks or bags under aseptic conditions.

1.2.1 Selection and preparation of containers for the sampling of foods contained in non-individual packages

- a) Use flasks or bags with leak-proof caps, made from non-toxic material approved for food contact and, preferably, autoclavable or pre-sterilized. The use of glass flasks or containers is not recommended due to the risk of breakage, contamination of the sampling environment with pieces of broken glass and loss of sample material.
- b) Choose flasks of appropriate size for the amount of food to be collected. To determine the quantity of sample to be collected, consider that each sample unit should contain, at least, twice the number of analytical units that will be used in the tests and, preferably, three or four times that amount (to allow for proper separation of counter-samples and prevention of possible spills or losses). Also consider that only three-quarters of the sampling flasks capacity should be filled with the sample (to prevent overflow and to allow proper mixing of sample before withdrawing the analytical units).
- c) Non-pre-sterilized flasks and utensils that will be used to collect food samples (such as spatulas, spoons, scissors, tweezers, openers, corer samplers, etc.) should, preferably, be sterilized individually in

an autoclave at $121 \pm 3^\circ\text{C}$ for at least 15 minutes or in a sterilizing oven at $170 \pm 10^\circ\text{C}$ for at least 1 hour (ISO 7218:2007/Amd.1:2013). Some other methods may be used as an alternative, such as flame sterilization, immersion in ethanol and alcohol combustion, and treatment(s) using disinfectant solutions. In the latter case, only disinfectants approved for use on food-contact surfaces should be used, in strict adherence to the manufacturer's instructions and followed by 12 rinsing cycles with sterile distilled water to remove all residues. Non-sterile flasks or bags showing – after having been subjected to an internal surface washing test – counts of viable microorganisms smaller than 1 CFU/mL of their holding capacity, may be used directly without previous sterilization.

1.2.2 Procedures for the sampling of foods contained in non-individual packages

- a) Before starting to collect the sample unit, the whole mass of the food should be thoroughly mixed, to ensure that the microorganisms will be evenly distributed throughout the food. Next, using appropriate utensils or instruments, withdraw the amount of product necessary to compose or complete the sample unit.
- b) If it is not possible to thoroughly mix the food mass before initiating sampling, portions should be taken from different parts of the content, until obtaining the amount of product appropriate to compose or complete the sample unit. Avoid withdrawing portions of the regions close to the surface or opening of the tank or container.
 - b.1) To collect powder samples from different parts of tanks or large packages, corer samplers or vertical double-tube samplers, long enough to reach the center of the food mass, may be used. A different sterile sampler or sampling device should be used for each sample unit to be collected, or the instrument should be sterilized between one sampling operation and the next.
 - b.2) To compose or complete a sample unit with portions taken from different points of foods that consist of one large solid piece, sterile knives, tweezers and forceps should be used to cut the food into smaller pieces.

- b.3)** In the case of large blocks of frozen foods, such as frozen fish blocks and frozen seafood blocks, volumes of frozen liquid egg, etc., the most adequate procedure is to use an electric drill (with a previously sterilized drill bit) in combination with a sterile funnel. Insert the drill bit in the funnel (the lower opening inside diameter of which should be only slightly greater than the diameter of the drill bit) and position the bit onto the point of the block from which a sample is to be taken. Turn on the drill and scrapings of the frozen food will move towards the surface and accumulate in the funnel, from where they can be transferred to an adequate collecting flask.
- b.4)** When samples are collected using faucets or tubes, the outer part of the outlet should be cleaned with ethanol 70% and sterilized by flame, if the material is fire resistant. The initial amount of product should be discarded before starting collection of the sample material. This will wash out the pipe and remove any accumulated dirt or residue particles.
- b.5)** For the sampling of margarine and similar products ("spreads") ISO 6887-4:2017 recommends removing the external layer (3 to 5 mm) and withdraw the sample units using a previously sterilized corer sampler. Insert the instrument diagonally, without reaching the bottom, rotate it in a complete circle and pull the sampler out, lifting out a conical portion of the product.
- c)** Remember that the external surface of collecting flasks and bags is not sterile. For that reason, do not hold flasks or bags directly above the mass of food, as contaminants may fall or otherwise be introduced into the product. Likewise, never insert a collecting flask directly into the product, but use an appropriate utensil instead to withdraw the sample units.
- d)** When withdrawing the collecting instrument filled with collected product, do not hold it above the other pre-sterilized instruments, because spatters of the food may contaminate the instruments that will be utilized later on.
- e)** Open the collecting flasks or bags only as far or wide enough to insert the product and close/seal immediately.
- f)** Do not touch the internal surface of collecting flasks or bags and their respective caps or closures.
- g)** Contaminated foods may contain microorganisms that are harmful to health. These samples should be collected by staff who are well trained in the handling of microorganisms and who are aware of the care required for protecting their health and safety. In case of doubt, each sample should be treated as if it were contaminated.

1.2.3 Sampling of foods involved in foodborne diseases

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that samples of all suspected foods should be collected and analyzed as soon as possible. However, it is of no use to collect samples that have undergone temperature abuse or that are already in a state of partial deterioration. The results of such analyses will be of no or little use to the conclusions of the investigation. If there are not any leftovers from suspected meals, one of the following alternatives may be tried: collect samples from similar meals, prepared at a later point in time but under the same conditions, collect samples of the ingredients and raw materials used in the preparation of the suspected meals and collect all containers and cooking utensils used to hold or prepare the suspected meals.

1.2.4 Sampling of water

Chapter 60 of the 5th Edition of the *Compendium* (Robin and Feng, 2015) deals with the collecting of samples of bottled water, which is considered a food by the Codex Alimentarius. These samples must be collected from their original sealed packaging. If there is any interest or need to collect smaller volumes from packaging of greater holding capacities, the entire content should be homogenized by inverting the packaging several times in quick succession. Next, the mouth or outlet should be disinfected with ethanol 70% and, under aseptic conditions, the seal broken open with a sterile or flame-sterilized knife or pair of scissors. Do not collect but dispose of the initial volume or runoff and then collect the sample in an adequate sterile flask.

To collect other kinds of water, section 9060A of the 22nd Edition of the *Standard Methods for the*

Examination of Water and Wastewater (Hunt, 2012) provides the following guidelines:

To collect samples from faucets or pipes, clean the external area of the outlet with a solution of 100 mg/L sodium hypochlorite or ethanol 70%, in addition to flame-sterilizing it if it is made of fire-resistant material. Open the faucet completely and let the water run for approximately 2 to 3 minutes to flush out any debris or impurities and clean out the piping system. Reduce the flow of water to collect a sample without spilling water droplets out of the collecting flask.

To collect water from wells or cisterns with a pump, the water should be pumped out for at least 5 to 10 minutes to allow the temperature of the water to stabilize before starting the actual sampling. In case there is no pump available, collecting flasks should be prepared by attaching a weight onto the base or bottom and then introducing the flask directly into the well. Care is needed not to contaminate the sample with material and impurities that may have accumulated onto the surface of the water.

To collect water from rivers, lakes or water reservoirs, hold the collecting flask by its base and then lower it into the water until it is totally immersed and covered by the water surface with the mouth of the bottle turned downward. Turn the mouth of the flask into the direction of the water flow with a slightly upward slope, so that the water will be retained. If there is no water flow or current, push the flask forward horizontally, in the direction opposite to that of the hand.

Samples of chlorinated water should have any residual chlorine neutralized immediately after the samples are taken, to immediately eliminate its bactericidal effect against the microbiota present. To that purpose, the collecting flasks should have 0.1 mL of a 3% sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) solution added (before sterilization) for each 100 mL of sample to be collected. This amount is sufficient to neutralize 5 mg of residual chlorine per liter of sample. In situations in which the concentration of residual chlorine exceeds 5 mg/L, utilize 0.1 mL of a 10% sodium thiosulfate solution for each 100 mL sample. This quantity is enough to neutralize 15 mg residual chlorine per liter of sample. Sterile plastic bags or flasks, which are commercially available and already contain sodium thiosulfate, may also be used. If the sample is collected and sent to the laboratory by the interested person, without previous neutralization of the chlorine, a sterile sodium thiosulfate solution should be added immediately upon arrival of the sample, under aseptic conditions.

1.3 Transportation and storage of samples until analysis

As a general rule, food samples should always be transported and stored in exactly the same way and under the same conditions the food is transported and stored until marketed. The guidelines below should be followed to ensure the integrity of the product until the time of analysis.

1.3.1 Foods with low water activity

Foods with low water activity (dehydrated, dried or concentrated), which are microbiologically stable, may be transported and stored at room temperature, although they should be protected against moisture.

1.3.2 Frozen foods

Frozen foods should be transported and kept frozen until the time of analysis. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends storage at -20°C . ISO 7218:2007/Amd.1:2013 recommends -15°C , preferably -18°C . The transportation should be carried out in Styrofoam boxes with dry ice with certain precautions and care. The product should not come into contact with the dry ice because the absorption of CO_2 may change the pH. If the lid does not make the packaging airtight and gas proof and/or if the packaging is gas permeable and/or becomes fragile or brittle at low temperatures, a secondary packaging should be used. Generally, wrapping in thick paper or paperboard is sufficient to avoid this problem. Labels and tags used to identify the samples should be waterproof, smudge proof and fade proof to avoid the loss of important data.

1.3.3 Refrigerated foods

Refrigerated foods should be transported and kept under refrigeration from the moment they are collected until the time of analysis. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends, as a general rule, that these samples be transported and stored at a temperature between 0°C and 4.4°C with a maximum time interval of 36 hours between sampling and analysis. If it is impossible to perform the analysis within 36 hours, the samples should be frozen or retained at

refrigeration temperatures for up to 18 hours, depending on the type of product, the reason for analysis and the type of analysis. ISO 7218:2007/Amd.1:2013 recommends that this kind of food sample be transported at a temperature between 1°C and 8°C and stored at $3 \pm 2^\circ\text{C}$, with a maximum interval of 36 hours between sampling and analysis (24 hours in the case of highly perishable samples). In case it is impossible to perform the analysis within the maximum time interval stipulated, the samples should be frozen and kept under the same conditions as those described for frozen samples (-15°C , preferably -18°C), provided freezing does not interfere with recovery of the target microorganism(s) (see the exceptions below).

Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that transportation be carried out in Styrofoam boxes containing ice. The *Compendium* further recommends the use of reusable gel ice packs, to avoid liquid from accumulating inside the boxes. If gel ice is not available, common ice may be used, provided it is pre-packed in plastic bags. Tightly closed Styrofoam boxes, with ample space inside for ice, in amounts sufficient enough to cover all sample-containing flasks, can keep the samples at appropriate refrigeration temperatures for up to 48 hours, in most situations. As a general rule, these samples should not be frozen, and for that reason, the use of dry ice inside the Styrofoam boxes is not recommended. If the transport of the samples requires a prolonged period of time, making the use of dry ice necessary, the sample packages should not come into direct contact with the dry ice packs, to avoid freezing. Labels and tags used to identify the samples should be waterproof, smudge proof and fade proof to avoid losing important data.

Exceptions: For certain microorganisms, differentiated rules apply, specified in the specific chapters. Samples of shellfish should be analyzed within 6 hours after sampling, and should not be frozen (Taylor *et al.*, 2015). Samples of refrigerated liquid egg should be analyzed, if possible, within 4 hours after sampling, and should not be frozen (Ricke *et al.*, 2015). Samples of non-heat-treated fermented or acidified products of plant origin should be stored under refrigeration for no longer than 24 hours, and should not be frozen (Pérez-Díaz *et al.*, 2015). ISO 6887-3:2017 recommends that the transport of raw fish, crustaceans, cephalopods, bivalve mollusks, gastropods, tunicates and echinoderms be done between 0°C and 10°C , without freezing. Laboratory samples shall be stored at $3 \pm 2^\circ\text{C}$, and microbiological examination should be initiated within 24 hours of collection.

1.3.4 Commercially sterile foods in sealed packages

Commercially sterile foods in sealed packages under normal circumstances may be transported and stored at room temperature, and should be protected against exposure to temperatures above 40°C (ISO 7218:2007/Amd.1:2013). Samples of bottled carbonated soft drinks, sold at room temperature, may also be transported and stored under these same conditions. Blown packages should be placed inside plastic bags due to the danger of leakage of materials of high microbiological risk. Transportation and storage can be carried out under refrigeration, to prevent explosion. However, if there is any suspicion of spoilage caused by thermophilic bacteria, refrigeration is not indicated because it may destroy vegetative cells (Parkinson and Francis, 2015).

1.3.5 Water samples

For water samples Chapter 60 of the 5th Edition of the *Compendium* (Robin and Feng, 2015) recommends that bottled water in its original, sealed packaging may be transported and stored at room temperature, without the need of refrigeration. Water contained in opened packages or water samples transferred to other containers should be transported and stored under refrigeration (temperature not specified). The samples should be analyzed within an interval of preferably 8 hours, but not exceeding 24 hours.

For other types of water, part 9060B of the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012) provides the following guidelines:

- a) **Drinking water for compliance purposes.** Preferably hold samples at temperatures below 8°C (without freezing) during transit to the laboratory. Do not exceed a 30-hour holding time from collection to analysis for coliform bacteria. Do not exceed an 8-hour holding time for heterotrophic plate counts.
- b) **Nonpotable water for compliance purposes.** Hold samples at temperatures below 8°C (without freezing), during a maximum transport time of six hours.
- c) **Other water types.** Hold samples at temperatures below 8°C (without freezing) during transport and until time of analysis. Holding time should not exceed 24 hours.

1.4 References

- Hunt, M.E. (2012) Microbiological examination. In: Rice, E.W., Baird, R.B., Eaton, A.D. & Clesceri, L.S. (eds) *Standard Methods for the Examination of Water & Wastewater*. 22nd edition. American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF), Washington, DC, USA. Part 9000, pp. 9.1–9.224.
- ICMSF (International Commission on Microbiological Specifications for Foods) (ed) (1986) *Microorganisms in Foods 2: Sampling for Microbiological Analysis: Principles and Specific Applications*. 2nd edition. Blackwell Scientific Publications, Oxford, England.
- ICMSF (International Commission on Microbiological Specifications for Foods) (ed) (2002) *Microorganisms in Foods 7: Microbiological Testing in Food Safety Management*. Kluwer Academic & Plenum Publishers, New York, NY, USA.
- ICMSF (International Commission on Microbiological Specifications for Foods) (ed) (2011) *Microorganisms in Foods 8: Use of Data for Assessing Process Control and Product Acceptance*. Springer, New York, NY, USA.
- International Organization for Standardization (2013) ISO 7218:2007/Amd.1:2013. *Microbiology of Food and Animal Feeding Stuffs: General Requirements and Guidance for Microbiological Examination*. 3rd edition:2007, Amendment 1:2013. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-3:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 3: Specific Rules for the Preparation of Fish and Fishery Products*. 2nd edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-4:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 4: Specific Rules for the Preparation of Miscellaneous Products*. 2nd edition. ISO, Geneva, Switzerland.
- Parkinson, N.G. & Francis, K. (2015) Canned foods: Tests for cause of spoilage. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 62, pp. 805–821.
- Pérez-Díaz, I.M., Breidt, F., Jr., Buescher, R.W. *et al.* (2015) Fermented and acidified vegetables. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 51, pp. 697–718.
- Ricke, S.C., Jones, D.R. & Gast, R.K. (2015) Egg and egg products. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 46, pp. 633–643.
- Robin, L.P. & Feng, P. (2015) Bottled water. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 60, pp. 791–796.
- Salfinger, Y. & Tortorello, M.L. (eds) (2015) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA.
- Taylor, T.M., Sofos, J.N., Bodnaruk, P. & Acuff, G.R. (2015) Sampling plans, sample collection, shipment, and preparation for analysis. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 2, pp. 13–25.
- Wehr, H.M. & Frank, J.F. (eds) (2004) *Standard Methods for the Examination of Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA.



Taylor & Francis

Taylor & Francis Group

<http://taylorandfrancis.com>

2 Preparation of samples for analysis

Revision history

Figures 2.1 e 2.2 (revised) Figures revised according to ISO 17604:2015.

Item 2.2.2 (revised) The procedure recommended for homogenization and withdrawal of analytical units from frozen solid foods was revised according the 2nd edition of ISO 6887-1:2017 and ISO 6887-3:2017.

Item 2.3.3 (revised) The diluents recommended by different regulatory agencies for food analysis was revised according the new editions of publications and standards.

Item 2.4 (revised) The uncertainty of volume measurement allowed by ISO 6887-1:2017 is 2%. The resuscitation step recommended by ISO 6887-4:2017 before preparing the second dilution of hard and dry products and for low moisture products is about one hour at ambient temperature (18-27°C).

Annex 2.1. (revised) The procedures recommended by different regulatory agencies for homogenizing the content and withdrawal of the analytical unit of different types of foods for analysis was revised according the new editions of publications and standards.

Annex 2.2. (revised) Variations recommended by different regulatory agencies for the preparation of the first dilution of different types of foods for analysis was revised according the new editions of publications and standards.

2.1 Introduction

Most of the guidelines contained in this chapter were taken from the American Public Health Association (APHA), as described in the 5th Edition of the *Compendium of Methods for Microbiological Examination of Foods* (Salfinger and Tortorello, 2015). When different from or complementary to those of the *Compendium*, they were completed with information and recommendations from the 22nd Edition of the *Standard Methods for the Examination of Water and Wastewater* (Hunt, 2012), specific to the microbiological examination of water, the 17th Edition of the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004), specific to the examination of dairy products and several standards developed by the International Organization for Standardization (ISO 6887-1:2017; ISO 6887-2:2017; ISO 6887-3:2017; ISO 6887-4:2017; ISO 6887-5:2010; ISO 7218:2007/

Amd.1:2013; ISO 17604:2015), recommended for tests performed using ISO method(s).

The preparation of samples for analysis involves three steps: (1) homogenization of the content and withdrawal of the analytical unit, (2) preparation of the first dilution of the analytical unit and (3) the preparation of serial decimal dilutions for inoculation into or onto culture media.

Before starting procedures certain precautions are recommended, to ensure that all activities be conducted under aseptic conditions:

Make sure that the work area is clean and that all doors and windows are closed to avoid air currents.

Disinfect all working surfaces with an appropriate disinfectant (ethanol 70%, 500 ppm benzalkonium chloride solution, 200 ppm sodium hypochlorite solution or any other chlorine-based compound is adequate).

Wash and disinfect your hands with a disinfectant appropriate and safe for skin contact. Verify the

necessity or not to use gloves in the chapters specifically dealing with pathogen tests.

Work inside vertical laminar flow cabinets to prevent contamination of the sample by the environment and contamination of the environment and the analyst by the sample. In case a vertical laminar flow cabinet is not available, work in an area located as close as possible to the flame of a Bunsen burner, which, when working well, will produce a steady blue flame. When handling powdered samples, it is not recommended to work very close to the flame of a Bunsen burner. ISO 7218:2007 stipulates the use of a separated area or a laminar flow cabinet.

Avoid the formation of aerosols when opening tubes, flasks or plates after agitating or releasing the content of pipettes or flame-sterilizing inoculation loops.

Never use a pipette by mouth; use mechanical pipettes instead.

After use, place the pipettes and other utensils in disposable trays and not directly onto the surface of the bench.

All instruments and utensils used to open packages and withdraw analytical units (scissors, tweezers, knives, spatulas, etc.) must be previously sterilized (in an autoclave or sterilization oven) or immersed in ethanol 70% and flame sterilized at the time of use.

Before opening the packages, disinfect the external area with ethanol 70%, maintaining contact until the alcohol has fully evaporated. In the case of flexible packages, cut open with a sterile pair of scissors. In the case of rigid packages with a screw cap, unscrew and remove the cap aseptically. In the case of cans that come with an “easy open” lid with wide opening, open the can aseptically and remove the lid. In the case of cans without an “easy open” feature, use a sterile can opener. In the case of cans, glass containers, boxes and other packaging intended to be subjected to the commercial sterility test, differentiated guidelines should be followed, as described in a specific chapter. The objective of these procedures is to ensure the integrity of the sealing system, for later analyses of the package, if necessary. Observe and note any abnormality concerning either the package itself or its content, such as blowing, leakage, off-odors and/or strange or atypical appearance, the presence of foreign objects and so on.

2.2 Homogenization of samples and withdrawal of the analytical unit

The analytical unit is the amount of material withdrawn from a sample to be subjected to one or more tests. The

number of analytical units that should be withdrawn and the amount of material of each analytical unit depends on the number and types of tests that will be performed on the same sample. In general, the following items are necessary:

- a) **Analytical units for presence/absence tests with enrichment in specific broth.** One analytical unit is required for each test (*Salmonella*, *Listeria* and others). The quantity of material of each of these analytical units is defined in the chapters specifically dedicated to these tests.
- b) **Analytical units for tests requiring differentiated treatment of the sample.** One analytical unit is required for each test (commercial sterility, bacterial spore counts, thermoresistant mold counts and others). The quantity of material of each of these analytical units is also defined in the chapters specifically dedicated to these tests.
- c) **Analytical units for general quantification tests.** General quantification tests usually comprise total aerobic mesophilic or psychrotrophic counts and counts of yeasts and molds, lactic acid bacteria, enterococci, *Enterobacteriaceae*, coliform and/or *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Pseudomonas* spp. These tests are performed with the same analytical unit, which, most commonly, consists of 25 g or 25 mL of the sample. According to ISO 6887-1:2017 the minimum of 10 g is specified for enumeration tests in many specific standards. For qualitative tests the size is normally 25 g (or 25 mL), although alternative quantities can be used. Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends that the minimum amount or volume of the analytical unit be at least 50 g for solid foods and 10 mL, 11 mL or 50 mL for liquid products. However, in the specific chapters, the recommended amount for most cases is 25 g or less. For more information on these exceptions see Annex 2.2.

Before withdrawing the analytical unit(s), the content of the sample should be well homogenized to ensure that the portion to be removed will be representative for the material as a whole. The procedures to achieve good homogenization are different for liquid products, solid products and products with a predominantly surface contamination, as will be further specified in the following sections.

2.2.1 Procedure for homogenization and withdrawal of analytical units from liquid products

If the liquid product (viscosity not greater than that of milk) is filled in containers with enough inner space to allow for agitation, invert the packaging 25 times. If the container is filled to more than two-thirds of its inner space, invert the package 25 times in a 30-cm arc within 7 seconds. If there is not enough free space for agitation, then use a second, sterile container and transfer the sample from one container to the other, for three consecutive times. If foam is formed, let it subside by standing until totally dispersed. As for gasified samples (carbonated soft drinks and similar products), transfer the content to a sterile container with a wide mouth and, with the cap slightly open, agitate using a shaker until the gas is completely expelled (this step is unnecessary if the analytical unit is transferred directly to the filtration flask, in the tests using the membrane filtration method).

Withdraw the analytical unit with a pipette, inserting the tip of the pipette to a depth not greater than 2.5 cm below the surface of the liquid. The measurement should be volumetric and the time interval between the homogenization of the sample and the withdrawal of the analytical unit should not exceed 3 minutes. The *Compendium* (Taylor *et al.*, 2015) does not set a limit for the uncertainty of the measurement of the volume, which, according to ISO 6887-1:2017, should not be greater than 5%.

2.2.2 Procedure for homogenization and withdrawal of analytical units from solid or concentrated liquid products

In the case of solid or concentrated liquid products, follow the guidelines contained in Annex 2.1, which defines the procedures most appropriate for homogenizing and withdrawing the analytical unit of different types of foods. The *Compendium* (Taylor *et al.*, 2015) recommends that the uncertainty of mass or weight measurement be not greater than 0.1 g. ISO 6887-1:2017 recommends this measurement uncertainty not to exceed 5%.

If the sample is frozen, the *Compendium* (Taylor *et al.*, 2015) recommends thawing in the original packaging under refrigeration temperatures ($\leq 4.4^{\circ}\text{C}$) for no

longer than 18 hours. Alternatively, higher temperatures may be used, but not higher than 40°C , and for no longer than 15 minutes. In this case, frequent agitation of the sample is required to facilitate thawing. The use of a controlled temperature water bath and agitation is recommended. ISO 6887-1:2017 recommends thawing under refrigeration ($5 \pm 3^{\circ}\text{C}$ for no longer than 24 hours), in the original packaging. Alternatively, higher temperatures may be used (18°C to 27°C for no longer than 3 hours), but, unlike the *Compendium*, ISO 6887-1:2017 does not recommend defrosting in water bath or under running cold water, as this can result in contamination of the sample if the packaging is not completely watertight. In the case of large blocks of frozen foods, which cannot be thawed under the conditions described above, the procedure recommended by ISO 6887-1:2017 can be used: with an electric drill (fitted with a previously sterilized drill bit) or a hand drill, make holes in several points of the piece. Set the speed of the drill to not more than 900 rpm to avoid fusion or dispersion of the shavings. Using a sterile spatula, collect the shavings in a sterile container or plastic bag, from which the required number of analytical units can be taken. The entire sampling operation shall not cause a significant increase in the temperature of the sample. For frozen fish, crustaceans, mollusks, tunicates and echinoderms, ISO 6887-3:2017 recommends either taking the test portion using a drill or defrost at ambient temperature (18°C to 27°C) for approximately 60 minutes but no more than 3 hours.

If the sample is heterogeneous, consisting of different layers, each of which is of a distinct and clearly different composition (filled cakes, pies, desserts and other ready-to-eat foods), the analytical unit should be put together using portions of the different layers, taking into account the actual proportion of each layer in the product. Alternatively, homogenize the entire content of the sample and withdraw the analytical unit from the macerate (ISO 6887-4:2017).

If the amount of sample sent for analysis is smaller than the analytical unit(s) required, the *Compendium* (Taylor *et al.*, 2015) recommends subjecting half of the available amount of sample material for analysis and reserving the other half as a counter-sample. If homogenization is done using a blender, the quantity of sample plus diluent (first dilution 10^{-1}) in the jar of the blender should be sufficient to cover the cutting blades of the apparatus. For meat products, ISO 6887-2:2017 recommends using all of the material for the tests.

2.2.3 Procedure for withdrawing the analytical unit using the surface swabbing technique

The surface swabbing technique applies to foods of which most microbial contamination is predominantly present or concentrated on the surface, such as bovine, swine, poultry and fish carcasses. It also applies to the analysis of the surfaces of pieces of equipment, tables, utensils and packaging.

Rubbing can be done with sterile swabs or, if the area to be sampled is large, with sterile sponges. This material can be purchased in individual, sterile packages. The sponges may be replaced by sterile cotton pads, prepared in the laboratory. The swabs may also be prepared in the laboratory, with wooden shafts of approximately 15 cm in length by 3 mm in diameter and the absorbent part in cotton measuring approximately 2 cm in length by 5 mm in diameter.

2.2.3.1 Swab sampling

Prepare tubes or flasks with 10 mL of an appropriate diluent. The *Compendium* (Taylor *et al.*, 2015) recommends 0.1% peptone water (PW) or Butterfield's phosphate buffer, and ISO 6887-1:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW). Remove the swab from its sterile package, holding it by the shaft at the edge opposite to the cotton tip. Moisten the cotton in the diluent, pressing it against the walls of the flask to remove any excess liquid.

Using a sterile frame of 50 cm² in size, delimit the area to be sampled, holding the frame firmly against the surface. Rub the swab with pressure, moving from left to right and then from bottom to top. Rotate the cotton swab tip continuously as you wipe, so that the entire surface of the cotton comes into contact with the sample. Upon completion of the rubbing or wiping, transfer the swab to the tube or flask containing the diluent, breaking off the hand-manipulated part of the wooden shaft against the inside of the flask tube, before immersing the remainder of the swab in the diluent.

Repeat this procedure one more time, covering the same sample surface area, using a dry swab this time. Place and keep the second swab in the same flask or tube containing diluent.

The liquid collected by the swabs can be used in general quantification tests or in presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. This procedure samples a total surface area of 50 cm² and each milliliter of

diluent, upon removal of the swabs, corresponds to 5 cm² of the sampled surface. Both the sampled surface area as the volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

For the swabbing of half bovine or swine carcasses using the same procedure, ISO 17604:2015 recommends sampling the points indicated in Figures 2.1 and 2.2. Use one swab for each point and, between one point and the next, immerse the frame in ethanol 70% and flame sterilize. The swabs may be placed and kept in one and the same

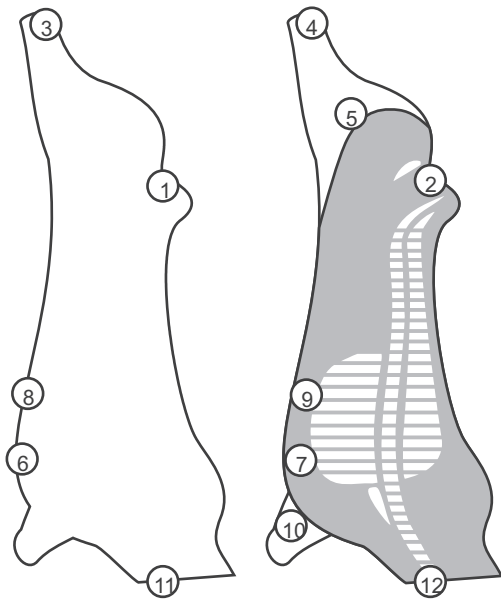


Figure 2.1 Points recommended by ISO 17604:2015 for swab sampling of bovine carcasses.

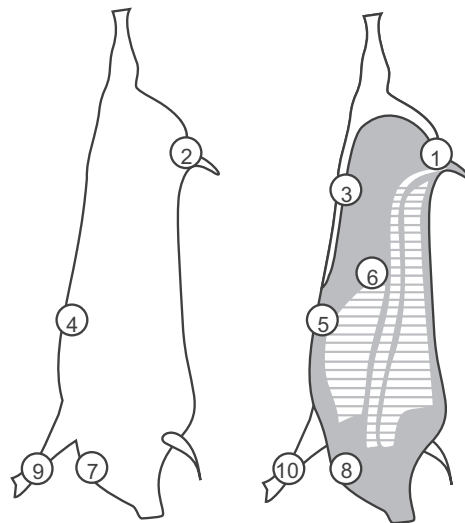


Figure 2.2 Points recommended ISO 17604:2015 for swab sampling of swine carcasses.

flask containing a total volume of diluent corresponding to a multiple of 10 mL diluent for each pair of swabs.

2.2.3.2 **Sponge swab sampling**

Prepare tubes or flasks with 25 mL of one of the diluents recommended for swabs. Open the plastic bag containing the sterile sponge (or cotton pad) and add an amount of diluent sufficient to moisten the sponge, without leaving behind any visible excess fluid. Hold the bag by its outside surface and massage the sponge to moisten it evenly. Thoroughly wash your hands before putting on a pair of sterile gloves and remove the sponge from the bag.

Using a sterile frame measuring 10 × 10 cm, delimit the area to be sampled by holding the frame firmly against the surface. Rub the sponge under pressure, moving it 10 times from left to right and 10 times from bottom to top. Upon completing this procedure, place the sponge back again into the bag and add the remainder of the diluent, until completing 25 mL.

The liquid collected by the sponges can be used in general quantification tests or in presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. This procedure samples a total surface area of 100 cm², and each milliliter of diluent, after the sponge is removed, corresponds to 4 cm² of the sample surface. Both the sampled surface area as the volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

2.2.4 **Procedure for withdrawing the analytical unit using the surface washing technique**

The surface washing technique is used for taking food samples of which most microbial contamination is predominantly present or concentrated on the surface, such as whole poultry carcasses, poultry cuts, fish, egg shells, grains, seeds, nuts and peanuts, which may be immersed in an adequate diluent contained in a sterile bag. The method is also used for the analysis of packages that can be closed and agitated with the diluent inside, for washing the package and collecting the sample to be examined.

2.2.4.1 **Procedure for washing poultry carcasses**

The following procedure is from MLG/FSIS (2017) to be used for the simultaneous examination of *Salmonella*

and other microorganisms. It is also recommended by ISO 17604:2015.

Aseptically drain excess fluid from the carcass and transfer the carcass to a sterile plastic bag. Pour 400 mL of buffered peptone water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for 1 minute (ca. 35 rpm). This is done by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other. Rock the carcass with a reciprocal motion in about an 18- to 24-inch arc, ensuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container. Use 30 ± 0.6 mL of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ± 0.6 mL of sterile BPW, and mix well. For analyses other than *Salmonella*, the dilutions can be made directly from the BPW rinse. Alternatively, the carcass may be rinsed in Butterfield's phosphate buffer instead of BPW. In this case, for *Salmonella* analysis add 30 ± 0.6 mL of double concentration BPW to 30 ± 0.6 mL of carcass-rinse fluid and mix well.

In this procedure each milliliter of washing liquid corresponds to the weight of the carcass divided by 400. For example, if the carcass weighs 1600 g, each milliliter of the washing liquid corresponds to 4 g of the sample.

2.2.4.2 **Procedure for washing other foods**

Transfer the sample to a sterile bag and weigh. Using the same diluents recommended for swabs, add to the bag the amount of diluent required for an initial 1:1 dilution (1 mL of diluent per gram of sample). Closing the mouth or opening of the bag with one hand, agitate the sample and massage the pieces inside the bag with the other hand from the outside, taking the necessary care and precautions to avoid that pointed or other protuberant parts come to pierce or puncture the package. In the case of grains, seeds, nuts and similar products, the sample may also be placed in a flask containing the diluent and agitated for 10 minutes in a laboratory shaker.

The liquid produced by this washing procedure may be used for general quantification tests or for presence/absence tests. In the second case, follow the guidelines and instructions in each of the specific chapters. In this procedure each milliliter of the washing liquid corresponds to 1 g of sample.

The volume of diluent may vary, in accordance with the needs or the characteristics of the sample.

2.2.4.3 Procedure for washing packages

This procedure is recommended for packages with a leak-proof cap or closure system. In the case of packages that do not have any cap/closure system or caps that are not leak proof, use the swabbing method.

Using the same diluents as those recommended for swabs, add to the package an amount of diluent sufficient to wash the entire internal surface by agitation (one-fifth of the package's holding capacity, for example). Close the package tightly and, with the hands agitate and swirl the package vigorously to remove the microorganisms adhered to the inner surface. Try to reach all the points of the inner surface, so as to guarantee complete removal of the contaminants present.

The liquid obtained by this washing procedure may be used for general quantification tests or for the presence/absence tests. In the second case, follow the guidelines and instructions in each specific chapter. In this procedure each milliliter of the washing liquid corresponds to the holding capacity of the package divided by the volume of the diluent. For example, if the holding capacity of the package is 500 mL and the volume of diluent is equal to 100 mL, each milliliter of the washing liquid corresponds to 5 cm³.

2.2.5 Keeping of counter-samples

After withdrawing the analytical unit(s), store the remaining material under the same conditions utilized prior to analysis (ISO 7218:2007). Perishable samples need to be frozen, but it is important to know that thawing of counter-samples for the purpose of repeating microbiological test(s) is not an acceptable practice, due to the possible death of part of the microbial populations that were originally present. In the case of frozen products, this problem can be resolved by thawing for analysis only the portion required for the test(s). The remaining quantity, which was not thawed, may be kept frozen to be used as a counter-sample for later repetitions of the test(s), if necessary. In the case of refrigerated products, there is no acceptable way to keep counter-samples without freezing. In case test(s) need to be repeated, the result(s) should be interpreted taking into account the fact that population(s) of the target microorganism(s) may have been reduced due to freezing.

In the case of samples the analytical unit of which has been collected by surface swab or sponge rubbing

technique or the surface washing technique, the part of the diluent retaining the contaminants and not used for subsequent microbiological testing should be frozen to serve as a counter-sample. Also in this case, it should be taken into consideration that the population(s) of the target microorganism(s) may have been reduced due to freezing.

The minimum time for keeping counter-samples is the time required for obtaining the results of the tests, but should be set at the discretion of the laboratory. The samples may be disposed of by throwing them in a dumpster, but samples deteriorated or suspected of containing microorganisms that are harmful to health should be decontaminated in an autoclave (121°C for 30 minutes) prior to final disposal (ISO 7218:2007).

2.3 Preparation of the first dilution of the analytical unit

To proceed with the analysis, the analytical unit must be diluted and homogenized with an adequate diluent, to allow inoculation into or onto culture media. The recommended diluents and initial dilution ratios vary with the type of sample and the type of test that will be performed, as described below.

2.3.1 Diluents for presence/absence tests

These tests are performed with dilution and homogenization directly in enrichment broth, specified in the corresponding chapters.

2.3.2 Diluents for tests requiring differentiated handling of the sample

Also for these tests the specific chapters should be consulted.

2.3.3 Diluents for general quantification tests

For these tests the recommendations showed at Table 2.1 apply.

Table 2.1 Diluents recommended by different regulatory agencies for food and water analysis.

Regulatory agency publication	Matrix to which it applies	Diluent recommended
APHA, <i>Compendium of Methods for Microbiological Examination of Foods</i> (Taylor <i>et al.</i> , 2015)	Foods	0.1% peptone water (PW) or Butterfield's phosphate buffer
APHA, <i>Standard Methods for the Examination of Water & Wastewater</i> (Hunt, 2012)	Water	0.1% peptone water (PW) or magnesium chloride phosphate buffer
APHA, <i>Standard Methods for the Examination of Dairy Products</i> (Davis and Hickey, 2004)	Milk and dairy products	Butterfield's phosphate buffer or magnesium chloride phosphate buffer
ISO 6887-1:2017	Foods	Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-2:2017	Foods pH below 4.5 Meat and meat products	Double-strength buffered peptone water [BPW] ² Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-3:2017	Fish and fishery products	Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-4:2017	Miscellaneous products	Saline peptone water (SPW) or buffered peptone water (BPW)
ISO 6887-5:2010	Foods pH below 4.5 Milk and dairy products	Double-strength buffered peptone water [BPW] ² 0.1% peptone water (PW) or buffered peptone water (BPW) or saline peptone water (SPW) or Ringer's solution quarter-strength or phosphate buffer acc. ISO 6887-5
ISO 6887-6:2013	Samples from the primary production stage (from farm to slaughterhouse)	Saline peptone water (SPW) or buffered peptone water (BPW)

The *Compendium* (Taylor *et al.*, 2015) recommends, for general use in the examination of foods, 0.1% peptone water (PW) or Butterfield's phosphate buffer.

Section 4.030 of the *Standard Methods for the Examination of Dairy Products* (Davis and Hickey, 2004) recommends, for general use in the examination of dairy products, Butterfield's phosphate buffer (called phosphate dilution water) or magnesium chloride phosphate buffer (called phosphate and magnesium chloride dilution water).

The *Standard Methods for the Examination of Water & Wastewater* (Hunt, 2012) recommends, for general use in the examination of water samples, 0.1% peptone water (PW) or magnesium chloride phosphate buffer (called buffered water).

ISO 6887-1:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of foods. For foods with pH below 4.5 recommends BPW double strength.

ISO 6887-2:2017 and ISO 6887-3:2017 also recommend saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of meat and meat products, and fish and fishery products.

ISO 6887-4:2017 recommends saline peptone water (SPW) or buffered peptone water (BPW) for general use in the examination of miscellaneous products and BPW double strength for foods with pH below 4.5.

ISO 6887-5:2010 recommends, for general use in the examination of milk and dairy products, 0.1% peptone water (PW), buffered peptone water (BPW), saline peptone water (SPW), Ringer's solution quarter-strength or phosphate buffered solution according ISO 6887-5.

There are special cases for which a different diluent is recommended. For more details on these exceptions see Annex 2.2.

2.3.4 How to prepare an initial 1:10 (10⁻¹) dilution

The initial dilution recommended for most samples is 1:10 (10⁻¹), obtained by adding *m* grams or milliliters of the sample to 9 × *m* milliliters of diluent. For example, for 25 g of sample, add 9 × 25 mL of diluent (225 mL). There are situations in which the diluent and the initial dilution are different. For more details on these exceptions see Annex 2.2.

2.3.5 How to prepare an initial dilution different from 1:10

In some special situations the first dilution is different from 1:10. To determine the volume of diluent necessary to obtain a predetermined 1: k dilution of the sample, use the $v = [(k \cdot m) - m]$ ratio. For example, to obtain a 1:50 dilution of an analytical unit of 10 g, add $[(50 \times 10) - 10]$ mL of diluent (490 mL). To obtain the same dilution for an analytical unit of 20 g, add $[(50 \times 20) - 20]$ mL of the diluent (980 mL).

2.3.6 Procedure for the preparation of the first dilution of liquid samples

In the case of liquid foods, transfer the analytical unit directly to tubes or flasks containing the amount of diluent necessary for a 1:10 dilution. Homogenize the sample with the diluent by agitation, inverting the container or package 25 times. To allow for perfect homogenization, use tubes or flasks with screw caps. They should be of a size sufficiently great to ensure that no more than two-thirds of their holding capacity is taken up by the analytical unit plus the diluent. There are special cases that require a different initial dilution. For further details on these exceptions see Annex 2.2.

2.3.7 Procedure for the preparation of the first dilution of solid or concentrated liquid samples

In the case of solid or concentrated liquid foods, transfer the analytical unit to a sterile homogenization flask or bag. Add to the sample the amount of diluent necessary to obtain a 1:10 dilution. Homogenize the analytical unit with the diluent, which can be achieved by manual agitation, shaking the flask in an inverted position 25 times through a 30-cm arc within 7 seconds (concentrated liquids, soluble powders), agitation in a peristaltic homogenizer (better known as stomacher) for 1 to 2 minutes (soft foods, pasty foods, ground or minced foods, poorly soluble powders) or in a blender (hard foods). In the case of homogenization using a blender, the *Compendium* (Taylor *et al.*, 2015) recommends using high speed during the first few seconds and low speed (8,000 rpm) for the remaining time, which should not exceed 2 minutes. If a more prolonged

homogenization is necessary, it is important to prevent excessive heating of the material. For that purpose, the *Compendium* (Taylor *et al.*, 2015) recommends cooling the diluent in an ice bath before use, while ISO 6887-4:2017 recommends not homogenizing for periods longer than 2.5 minutes. There are special cases that require a different initial dilution. For further details on these exceptions see Annex 2.2.

2.3.8 Procedure for the preparation of the first dilution of samples obtained by surface swabbing or surface washing

The diluent retaining the contamination collected with swabs, sponges or surface washing is, in itself, already the first dilution of the sample. The subsequent treatment of serial decimal dilution is performed using this suspension as point of departure. Since the initial dilution is not the standard 1:10 dilution, this difference must be taken into account when doing the final calculations of the results, as described in Chapters 3 and 4.

2.4 Serial decimal dilution of the sample

The preparation and inoculation of serial dilutions of the sample are required for quantitative tests, to reduce the number of microorganisms per unit of volume and to make it possible to count them. This series of dilutions is generally decimal or 10-fold for ease of calculation of final results.

The number of dilutions necessary depends on the expected level of contamination and should be such as to allow for, in plate counts, obtaining plates with numbers of colonies varying between 25–30 and 250–300 (see Chapter 3) or between 15 and 150 in yeast and mold counts. In counts by the most probable number (MPN) method, the number of dilutions must allow for obtaining positive tubes at the lowest dilutions and negative tubes at the highest dilutions (see Chapter 4).

According to the general procedure described by the *Compendium* (Petran *et al.*, 2015), the second dilution is to be initiated immediately upon completion of the first dilution. The duration of the complete procedure, from the preparation of the first dilution until inoculation of all culture media, should not exceed 20 minutes (except when described in case-specific chapters).

According to the general procedure described by ISO 6887-1:2017, the duration of the complete procedure should not exceed 45 minutes and the time interval between the end of the preparation of the first dilution and the beginning of the second and subsequent dilutions should not exceed 30 minutes (except when specified in specific procedures).

For hard and dry products and for low-moisture products, ISO 6887-4:2017 recommends a resuscitation step before preparing the second dilution. In general, leave the sample to rest at laboratory temperature (18°C to 27°C) for about 1 hour (20 to 30 minutes in case of flours, cereal grains and by-products and animal feeds).

In all cases in which volumes are transferred, the uncertainty of the measurement must not exceed 2% (ISO 6887-1:2017).

How to prepare the second dilution (10⁻²): Transfer aseptically 1 mL of the first dilution (10⁻¹) to 9 mL of diluent. The diluents are the same as those recommended for the first dilution. In the second dilution there are no special cases in which a different diluent is required from the one used to prepare the first dilution.

Do not dip the tip of the pipette to a depth of more than 1 cm when pipetting the volume from the first to the second dilution (ISO 6887-1:2017). If the first dilution does not contain suspended particles, the material may be agitated before transferring the volume from the first to the second dilution. If there are suspended particles, ISO 6887-1:2017 recommends not to agitate and wait until the suspended particles settle to the bottom before transferring the volume. In the case of viscous samples, which adhere to the internal wall of the pipette, ISO 6887-5:2010 recommends dispensing the volume and subsequently washing the pipette with diluent (by aspirating several times) to ensure that all the material be transferred to the second dilution.

How to prepare subsequent dilutions: Transfer 1 mL of the previous dilution to 9 mL of diluent. Before withdrawing the volume to be transferred, agitate the tube vigorously, inverting it 25 times in a 30-cm arc (within 7 seconds) or using a laboratory vortex mixer (15 seconds).

2.5 References

Davis, G.L. & Hickey, P.J. (2004) Media and dilution water preparation. In: Wehr, H.M. & Frank, J.F. (eds) *Standard Methods for the Examination of Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA. Chapter 4, pp. 93–101.

- Duncan, S.E., Yaun, B.R. & Sumner, S.S. (2004) Microbiological methods for dairy products. In: Wehr, H.M. & Frank, J.F. (eds) *Standard Methods for the Examination of Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA. Chapter 9, pp. 249–268.
- Frank, J.F. & Yousef, A.E. (2004) Tests for groups of microorganisms. In: Wehr, H.M. & Frank, J.F. (eds) *Standard Methods for the Examination of Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA. Chapter 8, pp. 227–248.
- Hayman, M.M., Pinkas, J.M. & Gray, R.J.H. (2015) Gums and spices. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 52, pp. 719–729.
- Hunt, M.E. (2012) Microbiological examination. In: Rice, E.W., Baird, R.B., Eaton, A.D. & Clesceri, L.S. (eds) *Standard Methods for the Examination of Water & Wastewater*. 22nd edition. American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF), Washington, DC, USA. Part 9000, pp. 9.1–9.224.
- International Organization for Standardization (2010) ISO 6887-5:2010. *Microbiology of Food and Animal Feeding Stuffs: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 5: Specific Rules for the Preparation of Milk and Milk Products*. 1st edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2013) ISO 7218:2007/Amd.1:2013. *Microbiology of Food and Animal Feeding Stuffs: General Requirements and Guidance for Microbiological Examination*. 3rd edition:2007, Amendment 1:2013. ISO, Geneva, Switzerland.
- International Organization for Standardization (2015) ISO 17604:2015. *Microbiology of the Food Chain: Carcass Sampling for Microbiological Analysis*. 2nd edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-1:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 1: General Rules for the Preparation of the Initial Suspension and Decimal Dilutions*. 2nd edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-2:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 2: Specific Rules for the Preparation of Meat and Meat Products*. 2nd edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-3:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 3: Specific Rules for the Preparation of Fish and Fishery Products*. 2nd edition. ISO, Geneva, Switzerland.
- International Organization for Standardization (2017) ISO 6887-4:2017. *Microbiology of the Food Chain: Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination, Part 4: Specific Rules for the Preparation of Miscellaneous Products*. 2nd edition. ISO, Geneva, Switzerland.
- Laird, D.T., Gambrel-Lenarz, S.A., Scher, F.M., Graham, T.E. & Reddy, R. (2004) Microbiological count methods. In: Wehr, H.M. & Frank, J.F. (eds) *Standard Methods for the Examination of*

- Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA. Chapter 6, pp. 153–186.
- MLG/FSIS (2017) Isolation and identification of *Salmonella* from meat, poultry, pasteurized egg, and siluriformes (fish) products and carcass and environmental sponges. In: Microbiology Laboratory Guidebook. [Online] Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC, USA. Available from: www.fsis.usda.gov/wps/wcm/connect/700c05fe-06a2-492a-a6e1-3357f7701f52/MLG-4.pdf?MOD=AJPERES [accessed 3rd February 2017].
- Njongmeta, N.A., Hall, P.A., Ledenbach, L. & Flowers, R.S. (2015) Acid producing microorganisms. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 19, pp. 229–236.
- Petran, R.L., Grieme, L.E. & Foong-Cunningham, S. (2015) Culture methods for enumeration of microorganisms. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 6, pp. 75–87.
- Ricke, S.C., Jones, D.R. & Gast, R.K. (2015) Egg and egg products. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 46, pp. 633–643.
- Salfinger, Y. & Tortorello, M.L. (eds) (2015) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA.
- Taylor, T.M., Sofos, J.N., Bodnaruk, P. & Acuff, G.R. (2015) Sampling plans, sample collection, shipment, and preparation for analysis. In: Salfinger, Y. & Tortorello, M.L. (eds) *Compendium of Methods for the Microbiological Examination of Foods*. 5th edition. American Public Health Association, Washington, DC, USA. Chapter 2, pp. 13–25.
- Wehr, H.M. & Frank, J.F. (eds) (2004) *Standard Methods for the Examination of Dairy Products*. 17th edition. American Public Health Association, Washington, DC, USA.

Annex 2.1 Procedures for homogenizing the content and withdrawal of the analytical unit of different types of foods

a) Powdered products: Homogenize the sample by vigorously agitating and inverting the package with your hands until well mixed or stir the content with a sterile spatula or glass rod. If there is not enough free space inside the package to allow for appropriate homogenization, transfer the whole content to a larger flask and proceed in exactly the same way (ISO 6887-5:2010). Withdraw the analytical unit with a sterile spatula.

b) Pasty or ground products: Stir the content with a sterile spatula or glass rod until well homogenized. Withdraw the analytical unit with a sterile spatula (Taylor *et al.*, 2015).

c) Yogurts with fruit pieces: For yogurt containing fruit pieces, the *Standard Methods for the Examination of Dairy Products* (Duncan *et al.*, 2004) recommends homogenizing the entire content of the sample unit in a blender for 1 minute before withdrawing the analytical unit.

d) Cheeses: The *Standard Methods for the Examination of Dairy Products* (Duncan *et al.*, 2004) recommends macerating the whole content of the sample unit (with a sterile spatula) and withdrawing the analytical unit from the mixture.

e) Very hard food products: ISO 6887-1:2017 and ISO 6887-4 2017 recommends grinding the sample until a homogeneous mixture is obtained. To avoid excessive heat in this process, do not homogenize for more than 1 minute at a time.

It is also possible to place samples inside a sterile plastic bag and beat the material with a sterile hammer, crumbling it into small bits and pieces. Mix well the fragmented sample, withdraw the analytical unit with a sterile spatula and, after addition of the diluent, keep the sample homogenized for 1 hour at 18°C to 27°C for the recovery of stressed cells (ISO 6887-4:2017).

ISO 6887-1:2017 and ISO 6887-5:2010 recommend when using a stomacher to place the sample and diluent in two or more sterile bags to prevent puncturing and possible sample spillage. When using rotary homogenizer do not homogenize for more than 2.5 minutes at a time.

f) Pieces of solid foods: Chapter 2 of the *Compendium* (Taylor *et al.*, 2015) recommends using a suitable instrument (knife, sterile scissors) to break or cut smaller pieces from various points in the piece until the required amount is obtained.

g) Eggs in the shell: For analysis of the internal content, Chapter 46 of the *Compendium* (Ricke *et al.*, 2015) recommends to remove any adherent material from the shell surface with a brush, immerse the eggs in ethanol 70% for 10 seconds and flame sterilize, or, alternatively, to immerse in 3:1 alcoholic solution of iodine for 10 seconds and allow to dry. Using sterile gloves open the eggs aseptically and place the internal content inside a sterile flask or bag, separating the yolk from the egg white if the analysis requires. Mix well and withdraw the analytical unit from the mixture.