Laboratory Methods and Services Committee Report

2023 AAFCO Annual Meeting
January 18, 8:00am-2:30pm, San Antonio, TX
January 19, 10:00am-11:00am San Antonio, TX

Committee Recommendations

None

Board Recommendations

None

Committee Participants

Members Present: Joshua Arbaugh (WV), Sally Flowers (KS), H. Dorota Inerowicz (OISC), Mary Koestner (MO), Teresa Riegel (FL), Kristi McCallum (co-chair/CO), Sharon Webb (co-chair/UKY), Dancia Wu (OISC), Dominika Kondratko, (CO), Robin Johnson (MT), Angela Swinford (FDA), Michele Swarbrick, (MN)
Advisors Present: Jenny Bailie (NutriQuest/AMA), Matt Nichols (Neogen), Lars Reimann (Eurofins), Ken Riter (PFI NPAL), Leo Schilling (Eurofins), Brian Fitchett (JM Smucker)
Virtual Attendees: Buddhika Galkaduwa (KS), Srinu Chigurupati (FDA), Christina Chrysogelos (FDA), Lawrence Novotny (Life member), Nancy Thiex (Life member), Brenda Snodgrass (AAFCO PTP), Ametra Berry (GA), Rebecca Moseley (AL), Tai Ha (NE), William Hoek (NY), Andy Crawford (Consultant AAFCO PTP), Jeff Horst (Agri King), Melanie Titley (CFIA)

Committee Report

Committee Activities

During the 2023 mid-year meeting, the LMSC heard a presentation by Nancy Thiex on Measurement Error and Sampling Methods followed by a presentation by Jennifer Combs on the results of the AAFCO Sampling Study conducted by the University of KY. Jona Verreth from the Montana Department of Agriculture gave a presentation on their laboratory's switch from the Fibertech M6 to the Ankom 200 for measuring Crude Fiber with several best practices when analyzing feed samples for Crude Fiber using the Ankom 200. APHL gave updates on APHL activities and resources for testing laboratories. Wednesday's meeting concluded with a presentation by Dancia Wu from the Office of the Indiana State Chemist on labeling issues with direct-fed microorganisms and the difficulties with testing for these microorganisms in animal feed. The LMSC held a panel discussion with State Regulatory representatives. This was a very good discussion that focused on communication between state laboratories and their regulatory customers. On Thursday, the LMSC met briefly to discuss training and training resources for feed testing laboratories. The AAFCO strategic plan was discussed and the LMSC agreed that a training program would be of great benefit to laboratories and especially with high staff turn-over and many experienced staff retire.

ACTION: Agenda approval
MOTION: Motion to accept the meeting agenda so moved by Joshua Arbaugh and Seconded by Sharon Webb. Motion passes.
ACTION: Refer the Pilot Sampling PT scheme project to the AAFCO PTP committee
MOTION: Sharon Webb made a motion to refer this Pilot Sampling PT scheme project to the PTP committee to address the details; Seconded by Sally Flowers. Motion passes.

Subcommittee Activities
No update was given by the Quality Assurance sub-committee at this meeting.
ACTION: None
MOTION: None

Committee Minutes
1) Welcome, Introductions, & Adoption of Agenda
2) Review of Committee Roster and Announcements
    a) Kristi McCallum reminded everyone that if you are a "member" of the FoodShield LMSC group, it doesn't necessarily mean you are a member of the committee. The FoodShield group was created to be able to post documents and send emails securely and easily.
3) Presentation: Measurement Error in Lab Prep & Sampling Methods (Nancy Thiex, Life Member)
    a) Refer to PowerPoint titled: Pilot PT for Lab Sampling posted on AAFCO website
    b) Nancy asked: How many people would be interested in participating in a routine Lab Sampling PT scheme? Fourteen labs would be interested and there was a lot of discussion on the details that need to be considered to set up the scheme. These discussions will take place in the PTP committee.
    c) Nancy asked: What else do we need to do beside participate in a Sampling PT? Suggestions included training such as Good Samples and preparation of materials to present to laboratory management on the importance of proper sampling and good sampling equipment.
4) Presentation: Results from AAFCO Sampling Study KY (Jennifer Combs, KY)
    a) Refer to the PowerPoint titled: AAFCO Sampling Study posted on AAFCO website
    b) Jennifer gave a background on the reason for the study which was to evaluate the efficacy of the current AAFCO Sampling procedures. An RFP was initiated in 2019. A summary of the sampling study results was reviewed and a brief history on the use of AAFCO's AVs was also provided. KY uses NIR to screen samples before determining if additional testing is needed. Status of Study: Raw data is complete, and they are working on getting the data to AAFCO's BOD. The board will decide what to do with the data once it's released from the Inspection and Sampling committee to them. There are some considerations with regards to getting it published before its released to the public.
5) Presentation: Making the switch from a Fibertech M6 to the Ankom 200 for measuring Crude Fiber: a not so boring tale (Jona Verreth, MT)
    a) Refer to the PowerPoint titled: Making the switch from a Fibertech M6 to the Ankom 200 for measuring Crude Fiber: a not so boring tale posted on AAFCO website.
    b) Jona cautioned that laboratories need to pay attention to high fat samples and suggested using a larger beaker that allows for stirring which helps to remove the fat.
6) Presentation: APHL Update (Robyn Randolph, APHL)
    a) Refer to the PowerPoint titled: Update on APHL Activities -Supporting Human & Animal Food Laboratories posted on AAFCO website.

b) Robyn covered recent and upcoming meetings and training opportunities. She reviewed the many resources available through APHL (e.g., quality, professional development, training courses). Robyn also gave an update on the status of the laboratory competency framework work group.

7) Presentation: Direct-Fed Microorganism for Animal Feed and Pet Food Guarantee Analysis Labeling Issues and Discussion (Dancia Wu, OISC)

a) Refer to the PowerPoint titled: Direct-Fed Microorganism for Animal Feed and Pet Food Guarantee Analysis Labeling Issues and Discussion posted on AAFCO website.

b) No significant difference between microorganism counts between AFIA 1996 plate method vs 3M petrifilm Lab (AOAC 2017) method.

Action Items

Responsible	Item	Action	Timing / Status
Co-chairs	Annual Hazards/Contaminants Survey	Revise and send survey to regulators for 2023	October 2023/Sent to AAFCO for email distribution
LMSC QA Sub-committee	QAQC Guidelines	Revise the QAQC Guidelines to align with ISO17025:2017	September 2022 – January 2023
LMSC	Training for Laboratory Staff	• Collect training resources for new AAFCO website/LMSC Training • Need volunteer labs to host trainings	January 2023 – January 2025

PILOT PT FOR LAB SAMPLING

AAFCO Midyear Meeting

January 18, 23023

San Antonio, TX

HISTORICALLY

- Much attention focused on analytical uncertainty
  - Method validation
  - PT
  - QC
- Little attention focused on error associated with sampling
  - No method validation
  - No PT
  - No QC

QUESTIONS

- Is it feasible to develop a PT for laboratory sampling?
- What can be learned from a PT for laboratory sampling?
- Ultimately, can a proficiency testing program for laboratory sampling advance the performance of sampling in laboratories?

SUMMARY OF EXPERIMENT

- Two feed test items were "manufactured" from common feed ingredients
- Shipped to labs for processing
- After processing, labs selected duplicate test portions for crude protein, NPN, fat, vitamin A, Ca, Zn, Cu
- Labs tested crude protein and returned test results
- Test portions for NPN, fat, vitamin A, Ca, Zn, Cu shipped to me
- Test portions sorted by analyte and shipped to volunteer labs who reported test results to me

FORMULATION OF TEST ITEMS

Test Items A and B were manufactured from the same ingredients, varying the masses of the ingredients to vary the concentrations of the analytes.

PT Item	Ingredients							
	Cracked Corn, g	Whole Flax Seed, g	$CaCO_3$, g	Zn, capsules @ 30 mg each	Cu, capsules@ 2 mg each	Urea, g	Vitamin A, capsules@ 10,000 IU each	Final Mass, g
A	600	340	40	2	2	40	6	1020
B	800	175	15	6	6	10	3	1000

CALCULATED ANALYTE CONCENTRATIONS

Analyte	Test Item A	Test Item B
Crude protein, %	22.7	13.2
Non-protein nitrogen, %	1.80	0.46
Crude fat, %	16.1	10.1
Calcium (Ca), %	1.59	0.62
Zinc (Zn), mg / kg (ppm)	83	201
Copper (Cu), mg / kg (ppm)	9.0	16
Vitamin A, IU/kg	60,000	30,000

TEST METHODS

Analyte	Test Method
Crude protein, %	1 lab using AOAC 976.05; 10 labs using AOAC 990.03
Non-protein nitrogen, %	AOAC 941.04
Crude fat, %	AOCS Ba 3-38
Calcium (Ca), %	AOAC 968.08; ICP-OES
Zinc (Zn), mg / kg (ppm)	AOAC 968.08; ICP-OES
Copper (Cu), mg/kg (ppm)	AOAC 968.08; ICP-OES
Vitamin A, IU/kg	HPLC
K, P, Mg, Fe, Mn, mg/kg (ppm)	AOAC 968.08; ICP-OES

OXFORD

Journal of AOAC INTERNATIONAL, 2022, 1–12

https://doi.org/10.1093/jaoacint/qsac117
Advance Access Publication Date: 26 September 2022
Research Article

SAMPLING METHODS

Piloting a Proficiency Testing Program for Laboratory Sampling of Animal Feed Materials

Nancy J. Thiex [1] and Charles A. Ramsey[2]

[1]Thiex Laboratory Solutions LLC, 46747 214th St., Brookings, SD, USA, [2]EnviroStat, Inc, PO Box 339, Vail, AZ, USA

Corresponding author's e-mail: Nancy.Thiex@gmail.com

Abstract

Background: Laboratory sampling is a significant source of error in feed testing. Proficiency testing programs such as the Association of American Feed Control Officials Proficiency Testing Program are an effective means of assessing error in and among analytical methods. However, all proficiency test items are comminuted and blended to control variability among items, effectively minimizing sampling error. Currently there is no mechanism for monitoring sampling error among laboratories.

Objective: The objective of this work was to investigate the feasibility of a proficiency testing program for laboratory sampling methods and provide insight into a program to advance the performance of sampling in laboratories.

Methods: The study involved the fabrication of identical feed test items from feed ingredients and shipping the uncomminuted materials to volunteer laboratories. The volunteer laboratories followed in-house procedures for selecting test portions for routine feed tests. Tests on all the test portions for a single analyte were performed by a single laboratory, so that the variability in test results could be attributed to laboratory sampling processes to select test portions.

Results: The average RSD, %, for Item A and Item B, respectively, were as follows: protein, 5.08 and 5.23; non-protein nitrogen, 8.90 and 16.6; crude fat, 3.45 and 5.67; vitamin A, 33.9 and 26.9; calcium, 21.9 and 23.6; zinc, 17.9 and 27.9; and copper, 17.4 and 27.9.

Conclusion: This study suggests that a proficiency testing program for laboratory sampling is feasible with manual manufacture of the test items, and data can be used to monitor laboratory sampling proficiency and also to compare the performance of different laboratory sampling methods.

Highlights: The data illustrates that each analyte has unique distributional and compositional heterogeneity, thus unique sampling error, even when multiple analytes are determined from a single test portion.

RAW DATA

Raw data available in JAOAC manuscript:
https://doi.org/10.1093/jaoacint/qsac117

Paper is open access.

		Test Result n=2	RSD %	Type of Analyte
Analyte	Test Item	Average	Average	
Protein, %	A	23.74	5.08	Intrinsic
	B	13.09	5.23	Intrinsic
NPN, %	A	1.82	8.90	Liberated
	B	0.49	16.60	Liberated
Fat, %	A	15.35	3.45	Intrinsic
	B	9.75	5.67	Intrinsic
Vitamin A, IU/kg	A	32295	33.9	Liberated
	B	20162	26.9	Liberated
Ca, %	A	1.24	21.85	Liberated
	B	0.49	23.55	Liberated
Zn, mg/kg	A	66.45	17.9	Liberated
	B	144	27.9	Liberated
Cu, mg/kg	A	10.17	22.36	Liberated
	B	14.68	19.43	Liberated
K, mg/kg	A	4342	6.0	Intrinsic
	B	4103	6.4	Intrinsic
P, mg/kg	A	3280	2.9	Intrinsic
	B	2925	3.8	Intrinsic
Mg, mg/kg	A	1863	2.7	Intrinsic
	B	1396	4.0	Intrinsic
Fe, mg/kg	A	44	23.2	Intrinsic
	B	29	15.0	Intrinsic
Mn, mg/kg	A	15.2	13.2	Intrinsic
	B	9.6	7.0	Intrinsic

SUMMARY OF DATA

Liberated analyte: Analyte is liberated from from the host material. These are added minerals and added NPN (urea). These are more difficult sampling problem.

Intrinsic: Analyte is inherent in the host feed material.

Analyte	Test Item	Low Test Result	High Test Result	Ave Test Result	RPD	Calc Conc	Recovery, %
Protein, %	A	21.4	26.47	23.74	21	23	105
	B	12.16	14.5	13.09	18	13	99
NPN	A	1.52	2.19	1.82	37	1.8	101
	B	0.402	0.725	0.49	66	0.46	107
Fat, %	A	14.37	16.35	15.35	13	16	95
	B	8.77	10.78	9.75	21	10	97
Vitamin A, IU/kg	A	14202	58819	32295	138	60000	54
	B	12793	30937	20162	90	30000	67
Ca, %	A	0.54	1.55	1.24	81	2	78
	B	0.23	0.64	0.49	84	1	79
Zn, mg/kg	A	34	80	66	70	83	80
	B	47	193	144	101	201	72
Cu, mg/kg	A	7.23	16.93	10.17	95	9	113
	B	7.39	20.83	14.68	92	16	92

LOW VITAMIN A RECOVERY

- Vitamin A test results were biased low and highly variable.
  - Recoveries were 54% for Test Item A and 67% for Test Item B.
- Two vitamin A capsules were tested as a check on the amount added.
  - The capsules, labeled at 10,000 IU/capsule, were determined at 10,700 and 11,500 IU/capsule.
- Since the capsules tested close to the label guarantee, the low bias to test results was due to laboratory sampling error.
- No amount of mixing can uniformly distribute the vitamin A product.
- Comminution of the entire laboratory sample and appropriately selecting test portion masses much greater than those provided by the participating laboratories can mitigate this error.
- Adding vitamin A to a PT item is challenging, as are other low concentration analytes, since the mass added to each PT item is small.

LESSONS LEARNED

- Difficult to manufacture feed!
- Choosing and characterizing feed ingredients is difficult
  - Obtaining proper particle size
  - Obtaining proper density to suspend well
- In this study, the calcium carbonate segregated and adhered to the plastic bags. (recovery ~79% with RSD ~ 23%)
- Labs reported problems with fines segregating and coating surfaces
- Automating the process will challenging

Test Item B Test Item A

OBSERVATIONS – DIVERSITY AMONG LABS IN APPROACH TO LABORATORY SAMPLING

- Only 3 laboratories performed comminution as a first step.
- The mass comminuted ranged from the entire test item (~1 kg) to just under 60 g.
- Five laboratories used rotary splitters, 5 used riffler splitters and 1 split manually.
- A laboratory using a riffler initially, and later used manual splitting following comminution.
- Final particle size ranged from 0.2 mm to 1.0 mm, with 5 laboratories using a final particle size of 0.75 mm.
- One laboratory had 3 steps to test portion selection, 5 laboratories had 4 steps to test portion selection, 4 laboratories had 6, and 1 had 7 steps.
- One laboratory generated duplicate analytical samples, and selected duplicate test portions from them (the only true duplicates)
- Only 1 laboratory performed no splitting operations.

Lab ID	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11
Step 1	Rotary split with 6 port	Riffle	Grind in a blender	Grind to 0.5 mm	Grind to 0.75 mm	Rotary split with 8 port	Split into 2 with riffler	Split into 2 with riffler	Rotary split with 8 port	Split into 2 with riffler	Split into 2 with riffler
Step 2	Combine 1 & 6 port	Grind 1/2 to 1 mm	Manually split	Mix	Rotary Split, 6 port	Combine 4 jars	Split into 2 with riffler	Split into 2 with riffler	Grind one jar to 0.75 mm	Split into 2 with riffler	Split into 2 with riffler
Step 3	Grind (~375 g)	Manually fill 3 jars	150 g further comminuted in a nut grinder	Rotary split	Test portions A and B from separate jars	Split with rotary spitter	Split into 2 with riffler	Split into 2 with riffler	Regrind to 0.2 mm	Grind one split to 0.75 mm	Split into 2 with riffler
Step 4	Test portion selection	Test portion selection	Manual splits	Test portion selection		Grind each jar to 0.75 mm	Grind one split to 0.75 mm	Split into 2 with riffler	Test portion selection	Test portion selection	Grind one split to 0.75 mm
Step 5			Test portion selection			Test portion selection	Test portion selection	Split into 2 with riffler			Test portion selection
Step 6								Grind			
Step 7								Test portion selection			
# of Steps	4	4	5	4	4	5	5	7	4	4	5
Comminute 1st			X	X	X						
Split 1st	X	X				X	X	X	X	X	X
Rotary	X			X	X	X			X		
Riffler		X					X	X		X	X
Manual		X	X								
Particle Size	NR	1 mm	No sieve used	0.5 mm	0.75 mm	0.75 mm	0.75 mm	NR	0.2 mm	0.75 mm	0.75 mm
Mass ground, g	330 g	500 g	1000 g	1000 g	1000 g	60 g	125 g	60 g	125 g	250 g	125 g

OBSERVATIONS FROM THIS STUDY

- Comparing the RSD of test results obtained from comminuting first to those from splitting first:
  - communication before splitting reduced the overall average random error by a factor of 2.4.
- Comparing the RSD of test results obtained for rotary spitting to those from stationary riffler splitting:
  - using rotary splitters reduced the overall random error by a factor of 1.7
- A combination of comminuting first and using a rotary splitter reduced random error by a factor of 3.4.

OBSERVATIONS FROM THIS STUDY

- Minerals were determined on a single test portion in an ICP profile, and each mineral demonstrated unique sampling error (unique RSD).
- One analyte (salt or protein or other) demonstrating a low RSD does not imply that the material can be assumed to be uniform (have low heterogeneity) for all analytes.
- Zn and Cu were added to the test items from a common source (analogous to a premix); however, RSDs for the two minerals were different.
  - Analytes added from a single premix will not have identical heterogeneity in the final feed material.

CONCLUSIONS

- Liberated analytes are more challenging to sample
- The uniformity of an intrinsic analyte cannot be used to predict uniformity for other analytes, especially liberated analytes. (RPD between high- and low-test results for intrinsic analytes averaged 25% while RPDs for liberated analytes averaged 81%)
- There is substantial room for improvement in laboratories' sampling techniques

CONCLUSIONS

- Even though there is more than one valid route to the same end, **a laboratory sampling proficiency testing program could facilitate a more consistent approach to laboratory sampling processes and direct laboratories toward the most accurate and most efficient practices**
  - How many labs would be interested? Is there a critical mass of interest? (Show of hands)

GOING FORWARD - LAB SAMPLING PT SCHEME

Able Laboratories is willing to prepare unground test items for AAFCO. Questions for potential participants.

1. How many test items per year? Quarterly?
2. What price would make it unacceptable?
3. Target start date? Third quarter of 2023 or 1st quarter of 2024?
4. What analytes would be of interest? Protein, NPN, Fat, Vitamin A, Ca, Zn, Cu, other minerals?
    a. Need to cover %,; 1000 mg/kg; 100 mg/kg; 10 mg/kg and lower, if possible.

GOING FORWARD - LAB SAMPLING PT SCHEME

Reporting considerations.

5. Critical information besides test results
   a) Test portion mass
   b) Information to replace "method code" – to define the process used.
      I. Comminute first or split first.
      II. Equipment used to split.
      III. Number of steps in process.
      IV. Mass comminuted.

GOING FORWARD - LAB SAMPLING PT SCHEME

6. Statistical approach
   a) Consensus value or formulated target concentration?
   b) Mechanism to compare results by test method to those obtained in the Animal Feed Scheme (in general or by lab?). Feasible or too complex? Each lab would have their own data to make in-house comparisons.

OPEN DISCUSSION

- Laboratory Sampling Process Improvement
  - Other than PT, what can we do?

- Are any labs interested in a hands-on dietary starch workshop? USDA ARS Laboratory in Madison, WI

Making the Switch From the FibertecM6 to the Ankom 200 for Measuring Crude Fiber

A Not So Boring Tale

AAFCO, January 2023

Bozeman Analytical Lab

- 8 FTE’s and 2 interns
- FY 2022: 2536 samples
- Regulatory Programs: Feed, Fertilizers, Pesticide Enforcement, Groundwater, Hemp and Organics
- MSU Ag Experiment Station: fee for service

Introduction

Crude Fiber:

- The insoluble residue of an acid hydrolysis followed by an alkaline one
- The plant cell wall components (including cellulose, hemicellulose and lignin), which are usually not or barely digestible, thus the portion of feed that is not energetically usable by the animals.
- Method was developed in 1867

ACTA AGRARIA DEBRECENIENSIS 2019-2
DOI: 10.34101/actaagrar/2/3670

Effect of feeds with different crude fiber content on the performance of meat goose

[1]Ádám Dobos – [1]Péter Bársony – [2]János Posta – [1]László Babinszky
[1]University of Debrecen, Department of Feed and Food-Biotechnology, Debrecen, Hungary
[2]University of Debrecen, Department of Animal Husbandry, Debrecen, Hungary
dobi1210@gmail.com

Overview

Fibertec method

1. 1 g of sample in crucible + add 1 g of sand
2. Place crucible on the cold extraction unit, add 20 mL of acetone to remove fat repeat this step 3 times
3. Transfer crucible to hot extraction unit, add 150 mL of simmering 0.255 N sulfuric acid solution.
4. Add a few drops of 1-octanol anti-foaming solution and digest at a moderate boil for 30 minutes

Ankom method

1. 1 g of sample into filter bag and seal bags
2. Soak filter bags in petroleum ether for 10 minutes and air-dry
3. Place bags in Ankom instrument and add room temperature 0.255 N sulfuric acid
4. Turn instrument on (agitate and heat) and extract samples for 40 minutes at 100°C

Fibertec method

5. Remove 0.255 N sulfuric acid solution
6. Add 150 mL of simmering 0.313 N NaOH solution
7. Add a few drops of 1-octanol anti-foaming solution and digest at a moderate boil for 30 minutes
8. Remove 0.313 N NaOH solution and rinse with hot water
9. Dry crucibles with fiber residue overnight at 110°C oven or 2 hours in a 130 °C oven
10. Weigh crucible with fiber residue and ash in muffle furnace.
11. Weigh crucible and ash residue

Ankom method

5. Turn instrument off, drain and rinse with water 2 times
6. Add room temperature 0.313 N NaOH
7. Turn instrument on (agitate and heat) and extract samples for 40 minutes at 100°C
8. Turn instrument off, drain and rinse with water 3 times
9. Soak in acetone for 5 minutes, dry and weigh
10. Place filter bag in crucible and ash in muffle furnace
11. Weigh crucible and ash residue

Ankom vs Fibertec

Ankom

Fibertec

Ankom vs Fibertec

Ankom

- In 2022: $10,925.00
- Low maintenance
- Up to 24 samples
- Acid/base solution: minimum 1500 mL/batch
- De-fat: minimum 350 mL pet ether/batch

Fibertec

- In 2005: $16,846.15
- Maintenance intensive, seals, glass manifold, moving parts
- Up to 6 samples at a time
- Acid/base solutions: 150 mL/sample
- De-fat: 20 mL acetone/sample
- Good precision/accuracy

	07.28.2022 F-57 Bags			08.03.2022 F-58 Bags			%RPD from actual result		
Sample No.	Average	Std Dev.	RSD%	Average	Std Dev.	RSD %	%RPD from actual result		
n=3							Sample AAFCO average/Expected Range (%)	%RPD 07.28.2022	%RPD 08.03.2022
AAFCO 201725-3	1.115	0.046	4.1	1.077	0.076	7.0	1.763	11.3	12.1
AAFCO 201925-3	27.51	0.177	0.64	27.823	0.572	2.1	29.49	1.7	1.5
AAFCO 202030-3	15.83	0.477	3.0	15.596	0.739	4.7	16.08	0.39	0.76
AAFCO 202130-3	3.978	0.475	11.9	4.324	2.700	62.4	3.878	-0.64	-2.7
AAFCO 202131-3	9.320	0.397	4.3	8.652	0.245	2.8	10.24	2.4	4.2
AAFCO 202293-3	4.687	0.259	5.5	5.777	0.826	14.3	5.196	2.6	-2.6

F-57 to F-58 filter bag comparison

Date	Sample Number	Result 1 (%)	Duplicate (%)	AVG (%)	RPD (%)
8/25/2022	AAFCO 202293	6.108	6.294	6.2	-3.0
8/25/2022	AC21877	2.945	3.051	3.0	-3.5
8/25/2022	AC21880	2.592	2.445	2.5	5.8
8/25/2022	AC22014	2.091	2.258	2.2	-7.7
8/25/2022	AC21766	2.146	1.070	1.6	66.9
8/25/2022	AC21778	7.079	6.682	6.9	5.8
8/25/2022	AC21820	8.384	9.191	8.8	-9.2
8/25/2022	AC21626	3.994	5.467	4.7	-31.1
8/25/2022	AC21627	4.605	4.127	4.4	10.9
8/25/2022	AC21628	3.877	3.830	3.9	1.2
8/30/2022	AAFCO 202293	7.247	6.088	6.7	17.4
8/30/2022	AC22104	3.360	4.801	4.1	-35.3
8/30/2022	AC22203	2.371	1.908	2.1	21.6

F-58 filter bags 40 min digestion time adjustment

Date	Sample Number	Result 1 (%)	Duplicate (%)	AVG (%)	RPD (%)
9/12/2022	AAFCO 202293	6.226	7.031	6.6	-12.1
9/12/2022	AC22159	2.94	3.601	3.3	-20.2
9/12/2022	AC22193	2.677	3.585	3.1	-29
9/12/2022	AC22203	2.638	3.352	3	-23.8
9/12/2022	AC22104	3.604	5.018	4.3	-32.8
9/12/2022	AC21877	4.114	4.444	4.3	-7.7
9/12/2022	AC21880	2.948	2.01	2.5	37.8

Crucibles with lid for ashing

2 x pet ether soaks

Date	Sample Number	Result 1 (%)	Duplicate (%)	AVG (%)	RPD (%)
9/19/2022	AC22159	1.761	0.4593	1.1	117.3
9/19/2022	AC22193	0.9713	1.855	1.4	-62.5
9/19/2022	AC22203	1.708	1.601	1.7	6.5
9/19/2022	AC22104	2.619	2.136	2.4	20.3
9/19/2022	AC21877	2.514	2.366	2.4	6.1
9/19/2022	AC21880	1.418	1.519	1.5	-6.9

Increase sealer setting

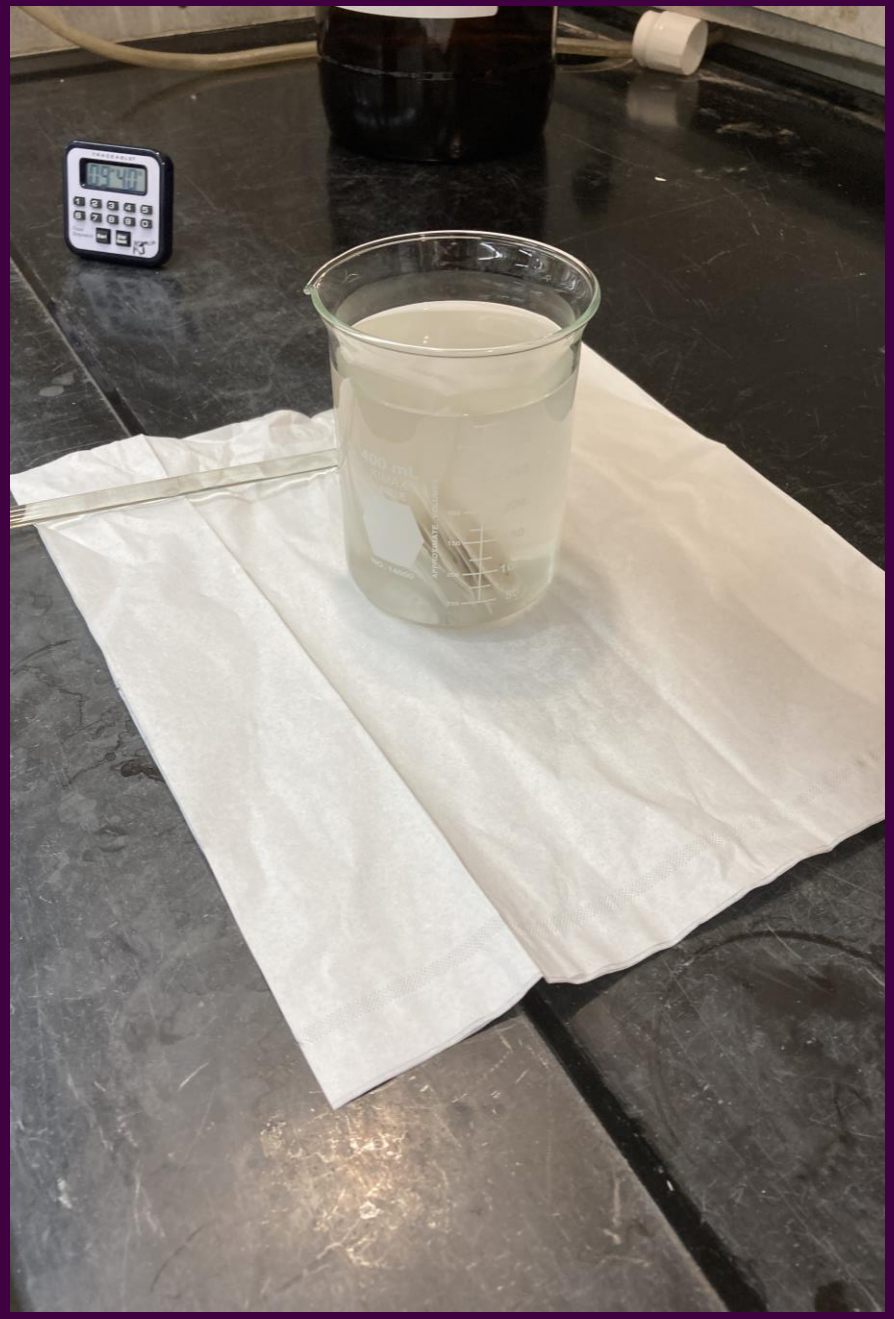
Date	Sample Number	AVG (n=3) %	Std. Dev	RSD (%)
12/5/2022	AAFCO 201925-1	30.18	0.436	1.45
12/5/2022	AC21627-1	4.827	0.534	11.1
12/5/2022	AC21820-1	9.445	0.188	1.99
12/5/2022	AC21871-1	2.460	0.688	28.0
12/5/2022	AC21946-1	3.210	0.302	9.41
12/5/2022	AC22194-1	4.221	1.205	28.6
12/5/2022	AC23037-1	20.20	0.579	2.86

ANKOM test results

Date	Sample Number	Result 1 (%)	Duplicate (%)	AVG (%)	RPD (%)
11/15/2022	AC21820	9.806	9.857	9.8315	-0.5
11/15/2022	AC21871	1.758	1.886	1.822	-7.0
11/15/2022	AC21946	2.425	2.787	2.606	-13.9
11/15/2022	AC21627	3.801	3.964	3.8825	-4.2
11/15/2022	AC22194	3.066	3.622	3.344	-16.6

MDA Fibertec vs MDA Ankom vs Ankom

		MDA Results		ANKOM Results	
Sample #	**Claim (%)**	**MDA Fibertec (%)**	**MDA ANKOM (%) n = 3**	**ANKOM (%) n = 2**	**RPD%**
AC21820	7.69-9.31	9.57	9.44	9.8	3.7
AC21871	1.58-2.42	1.50	2.46	1.8	-31.0
AC21946	2.52-3.48	1.97	3.21	2.6	-21.0
AC21627	2.52-3.48	3.67	4.83	3.9	-21.3
AC22194	2.52-3.48	2.73	4.22	3.3	-24.5



High tech fat removal!

Date	Sample Number	AVG (n=3) %	Std. Dev	RSD (%)	Claim Range
12/19/2022	AC22194	3.096	0.3391	11.0	2.52-3.48
12/5/2022	AC22194	4.221	1.205	28.6	2.52-3.48
10/17/2022	AC22194	5.284	1.097	20.8	2.52-3.48

Original collaborative study

TABLE 1 **Results of a collaborative study carried out in 2005.**

	Whole Corn	Cattle Feed	Alfalfa	Whole Soy	Poultry Starter	Calf Starter	Swine Feed	House Feed	Soy Meal	Pig Starter	Dog Food
Number of laboratories	11	10	11	10	11	11	9	10	11	11	10
Number of replicates	22	20	22	20	22	22	18	20	22	22	20
Mean	1.69	14.44	22.62	9.6	4.65	10.73	17.72	6.21	3.7	2.83	1.25
Reference method value[a]	2.05	14.23	22.67	9.57	4.4	10.7	17.4	6.43	3.73	2.85	1.45
Repeatability											
s_r	0.16	0.44	0.36	0.32	0.26	0.28	0.18	0.1	0.2	0.09	0.23
RSD_r	9.6	3.1	1.6	3.3	5.5	2.6	1.1	1.6	5.3	3.3	18.1
r	0.46	1.23	1	0.88	0.72	0.8	0.51	0.27	0.55	0.26	0.6
Reproducibility											
S_R	0.19	0.44	0.67	0.48	0.27	0.33	0.28	0.27	0.22	0.17	0.31
RSD_R	11.4	3.1	2.9	5	5.8	3.1	1.6	4.3	6	6	24.5
R	0.54	1.23	1.86	1.34	0.75	0.94	0.78	0.75	0.62	0.48	0.86

[a]Official Method Ba 6-84/AOAC 962.09.

Latest Ankom Test Results

Date	Sample Number	Result 1 (%)	Duplicate (%)	AVG (%)	RPD (%)	Claim Range	Crude Fat (%)
12/27/2022	AAFCO 201925	30.4934	30.3893	30.4	0.34	28.05-30.93	1.3
12/27/2022	AC23191	30.5456	30.9442	30.7	1.30	NA	NA
12/27/2022	AC23157	2.2643	2.2884	2.28	1.06	6.28-7.72	11.5
12/27/2022	AC23169	5.4083	6.0531	5.73	11.25	7.22-8.78	4.5
12/29/2022	AAFCO 201925	31.20	29.53	30.4	5.50	28.05-30.93	1.3
12/29/2022	AC23194	14.71	13.47	14.1	8.81	17.56-20.44	35.3
12/29/2022	AC23198	6.57	6.14	6.36	6.73	10.792-12.80	14.5
12/29/2022	AC23207	8.72	8.75	8.74	0.29	13.8-16.2	3.3
12/29/2022	AC23219	3.80	3.72	3.76	2.23	10.98-13.02	3.0

Date	Sample Number	AVG (n=3) %	Std. Dev	RSD (%)	Claim Range	Crude Fat (%)
12/19/2022	AC21776	1.79	0.177	9.9	1.58-2.42	3.1
12/19/2022	AC22194	2.93	0.336	11.5	2.52-3.48	17.5
12/27/2022	AC22995*	16.6	0.275	1.7	13.8-16.2	4.1
12/27/2022	AC23066	11.8	1.180	10.0	10.04-11.96	61

AC22995* fibertec result (n=4): 16.7

Conclusions

- High fat samples: cat food and some dog food samples
- Adjustment to AOCS Ba 6a-05 procedure to use a 600 mL beaker for batches with > 15 samples and include stirring of the samples in step 5. For batches with ≤ 15 samples a 400 mL beaker can be used. Soaking alone will not always work when dealing with high fat samples.

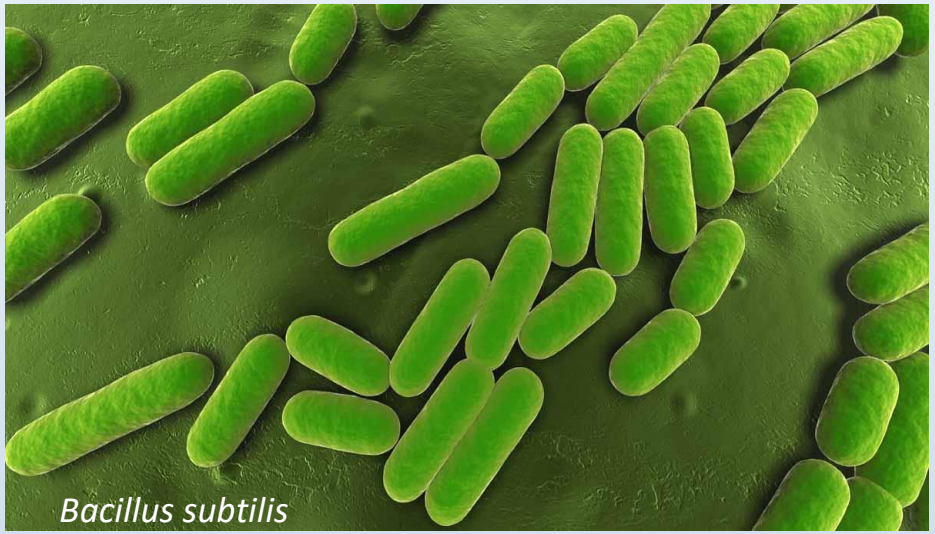
Thank you

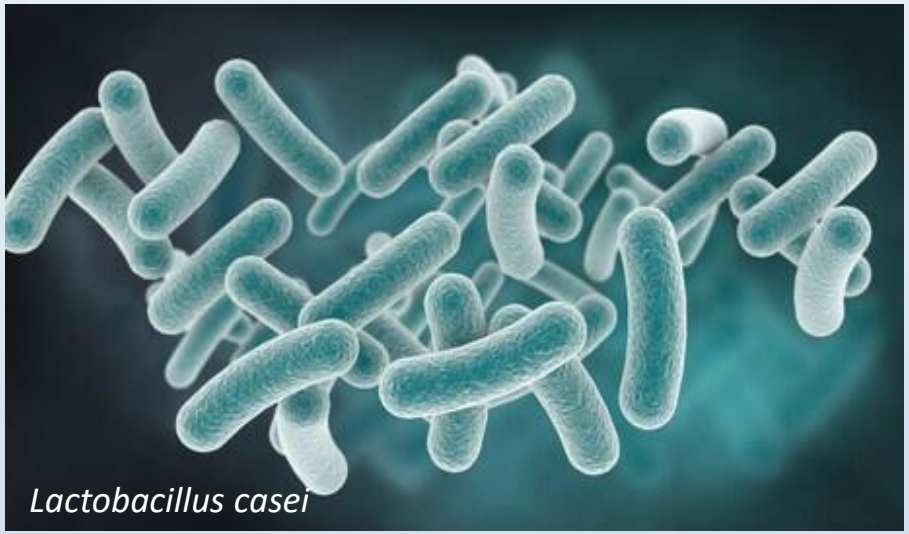
Robin Johnson
406-577-7919
robinjohnson@mt.gov

Jona Verreth
406-577-7918
jverreth@mt.gov

Direct-Fed Microorganisms for Animal Feed and Pet Food Guarantee Analysis Labeling Issues and Discussion

Dancia Wu
Microbiology laboratory
Office of Indiana State Chemist


Bacillus subtilis


Lactobacillus casei

Summary of the Presentation

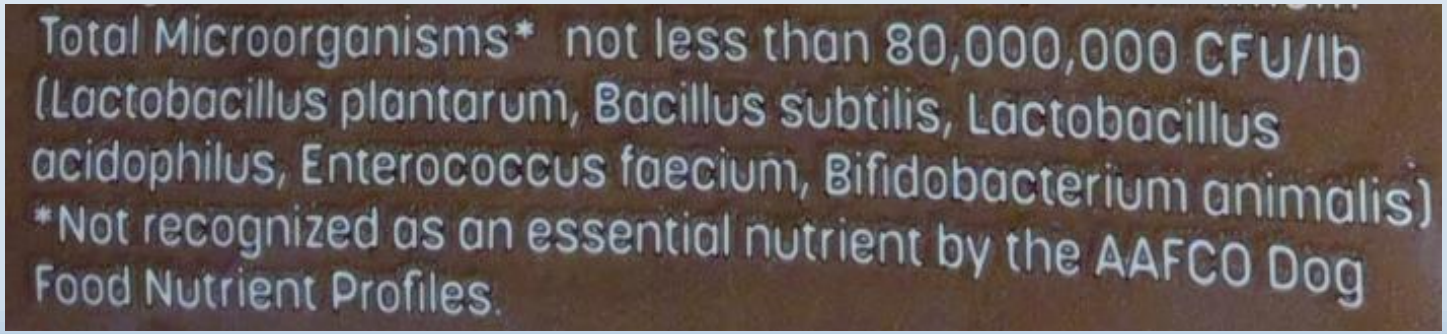
- What we proposed for direct-fed microorganisms labeling in animal feed and pet food
- AAFCO requirements of direct-fed microorganisms labeling for animal feed and pet food
  AAFCO Official Publication Regulation 9(b) and 4(g)
- Enumeration methods that we use and some methods that are published for microbial counts in animal feed and pet food
- Microbial count results from our lab and some detailed examples
- Suggestions and discussion

What we proposed for direct-fed microorganisms labeling of pet food and animal feeds

Separate labeling of the different classes (groups) of microorganisms guarantee analysis for animal feed and pet food Lactic Acid Bacteria, Bacillus, and Yeast/Mold

Total Lactic Acid Microorganisms* (Enterococcus faecium, Lactobacillus bulgaricus, Enterococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei)	not less than	100,000,000 CFU/lb
Total Bacillus Organisms* (Bacillus licheniformis, Bacillus subtilis)	not less than	7,000,000 CFU/lb

Lactic acid bacteria 5
Bacillus 2

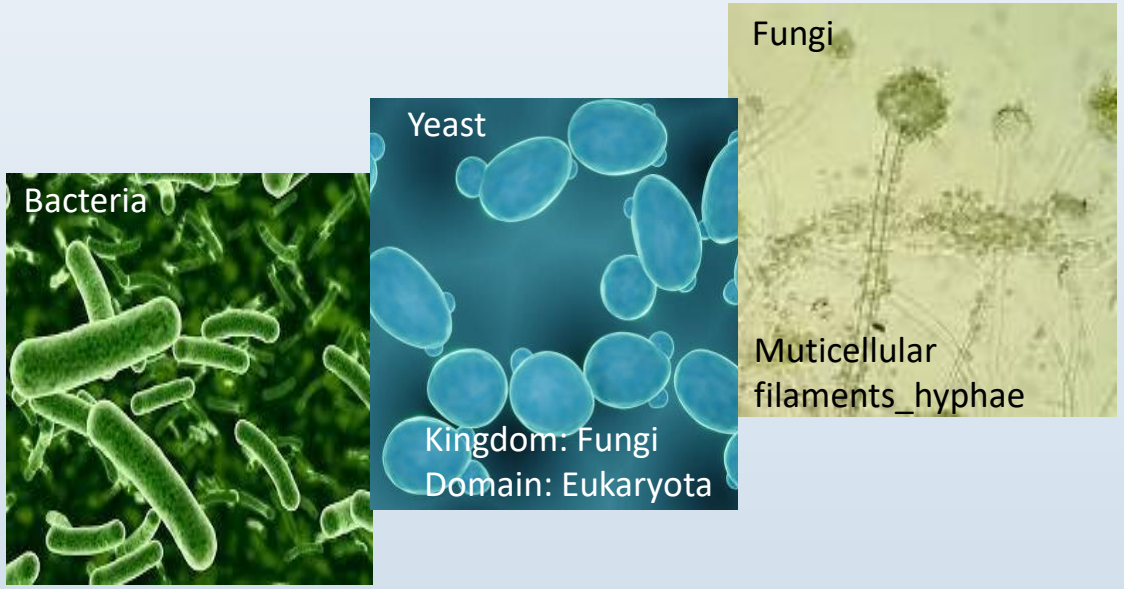


Lactic acid bacteria 4
Bacillus 1

Direct-Fed Microorganisms (AAFCO 2023 publication FDA-CVM approval safe for animal feed)

AAFCO 2023 Official Publication listed **45** direct-fed microorganisms were reviewed by the FDA , CVM, and found to present no safety concerns when used in direct-fed microbial products. Those 45 direct-fed microorganisms cross **13 genus**.

Bacillus* *6 (endospores)*
Bacteroides 4 (non endospores)
Bifidobacterium* *6 (LAB)*
Enterococcus* *4 (LAB)*
Lactobacillus* *12 (LAB)*
Leuconostoc 1 (LAB)
Pediococcus 3 (LAB)
Megasphaera 1 (Cattle only)
Propionibacterium 2 (PAB, LAB)
Rhodopseudomonas 1
Streptococcus 2
Saccharomyces 1 (Fungi)*
Aspergillus 2 (Fungi)



Most common groups of microorganisms in animal feed and pet food

Genus:	Lactobacillus	Enterococcus	Bifidobacterium	Bacillus	Aspergillus	Saccharomyces
Species:	*L. acidophilus,* *L. casei,* *L. plantarum*	*E. faecium* *E. lactis*	*B. bifidum* *B. infantis* *B. longum*	*B. subtilis,* *B. coagulans* *B. amyloliquefaciens* *B. licheniformis*	*A. oryzae* *A. niger*	***S. cerevisiae***
	Facultative anaerobic	**Facultative anaerobic**	**Anaerobic**	**Aerobic**	**Obligate aerobic**	**Facultative anaerobic**

**Bifidobacterium* is not included in the traditional Lactic Acid Bacteria due to its genetic unrelatedness, but the *Bifidobacterium* has a habitat that overlaps with LAB, and it has a metabolism that produces lactic acid as a primary end-product of fermentation.

There isn't a universal method to enumerate all microorganisms. Lactic acid bacteria, yeast/mold, and Bacillus request different growth media and incubation temperatures.

DIRECT FED MICROBIALS (DFMs)

AAFCO Official Publication Regulation 9(b) and 4(g), commercial feed has three direct fed microbial requirements.

1. The label should contain the statement “Contains a source of live (viable), naturally occurring microorganisms.”

2. The label guarantee should be consistent with Regulation 4(g). The units for the guarantee shall be stated in colony forming units CFU/g or CFU/lb, depending on the directions for use. A parenthetical statement should follow the guarantee, listing species in order of predominance.

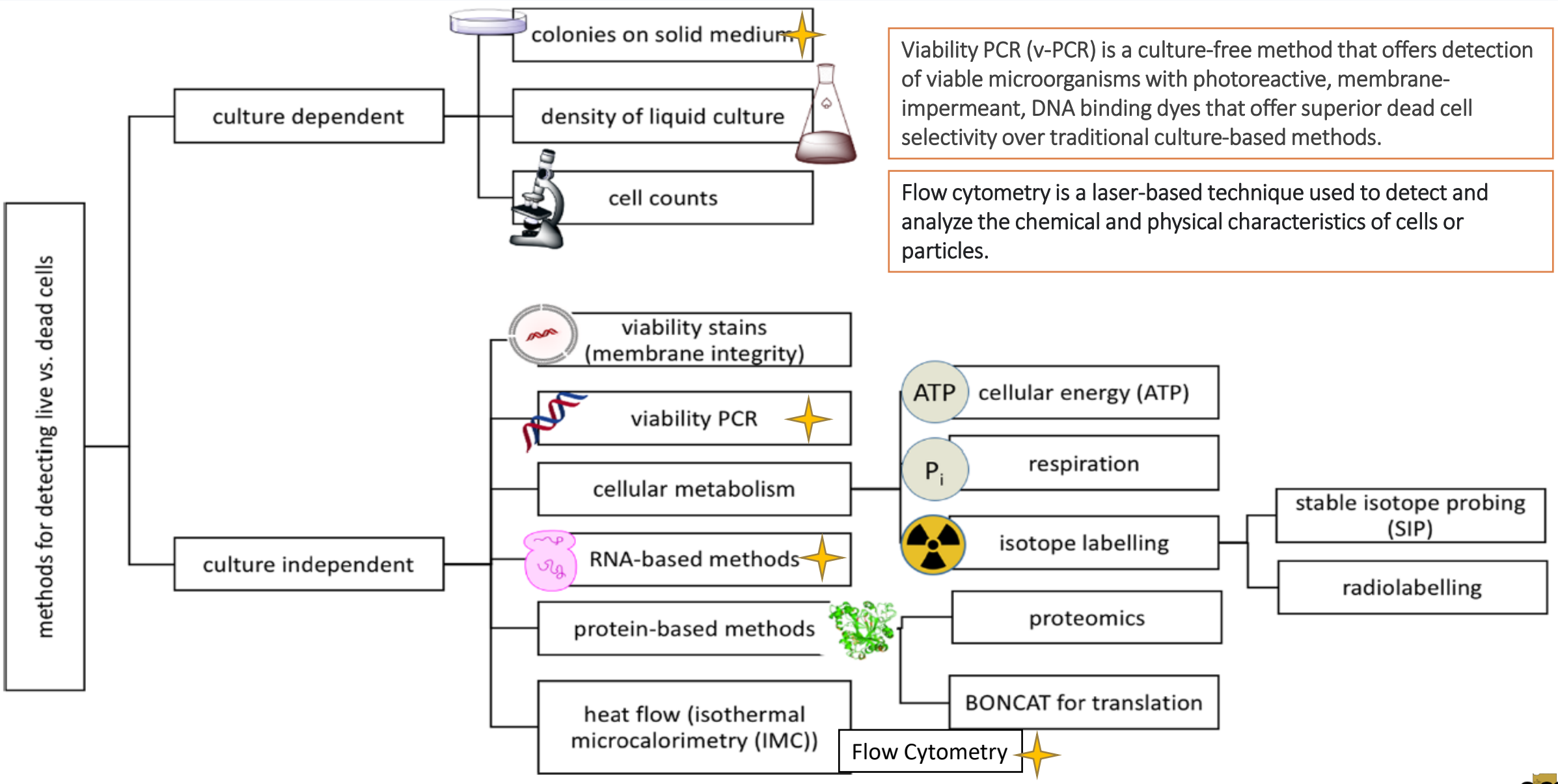
3. The ingredient(s) should meet the appropriate AAFCO fermentation product definition and be identified in the ingredient statement.

Fermentation Products is the product derived by culturing bacteria on appropriate nutrient media for the production of one or more enzymes, fermentation substances, or other microbial metabolites.

Guaranteed analysis formats for DFMs

Total microbial count, **minimum** ……..1.725 Billion CFU/lb (*Lactobacillus acidophilus, Bifidobacterium animalis, Bacillus subtilis, Bacillus licheniformis, Lactobacillus lactis*)

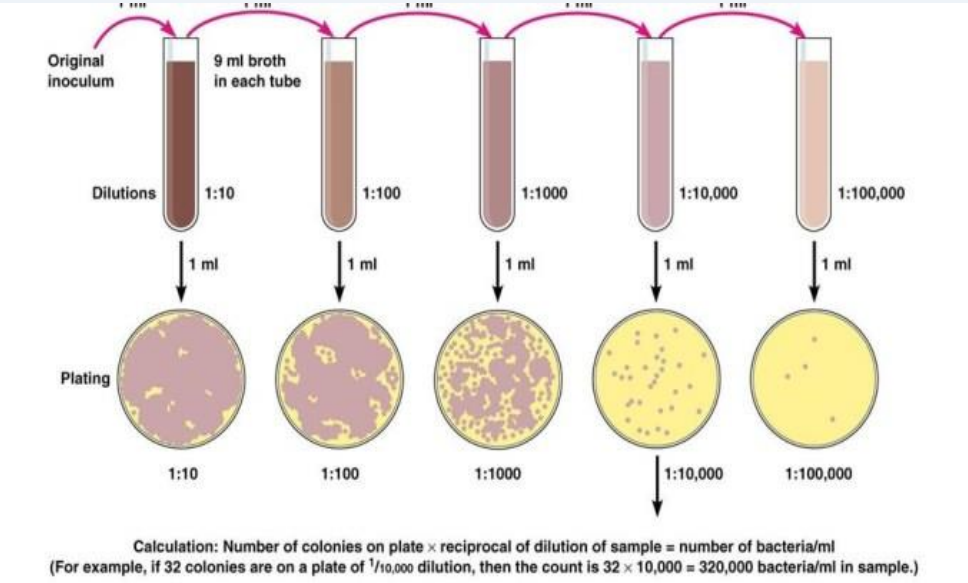
Schrodinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems
Microbiome 2017, Emerson et al.



Viability PCR (v-PCR) is a culture-free method that offers detection of viable microorganisms with photoreactive, membrane-impermeant, DNA binding dyes that offer superior dead cell selectivity over traditional culture-based methods.

Flow cytometry is a laser-based technique used to detect and analyze the chemical and physical characteristics of cells or particles.

Direct/Viable plate count methods



Extraction > Dilutions > Plating > Count colonies > Calculation CFU x dF

Advantages

1. It is sensitive method, since small numbers of microorganisms can be counted. A single cell can be detected.
2. It allows for inspection and positive identification of the microorganism counted.
3. Pure isolate can be further cultured for larger production.
4. Measurement of population of any magnitude.
5. No expensive instrument and materials required. It is easy to perform.

Limitations

1. Only living cells develop colonies.
2. Colonies develop only from those microorganisms for which the cultural conditions are suitable for growth.
3. Clumps or chains of cells develop into a single colony.
4. Not specific. Hard to identify species in closed family. Requires more identification tools.
5. Some species require long incubation time.
6. Some microbes are difficult to culture.
7. Viable but non-culturable (VBNC) bacteria will not grow in culture media.

Enumeration Methods of Microorganisms for Animal Feed and Pet Food
Total Microbial Count Determination (viable plate count method)

Total Yeast/Mold Count	Total Lactic Acid Bacteria Count	Total Bacillus Count
3M Petrifilm Rapid Yeast and Mold (RYM) Count Plate (sample-ready-culture-medium system containing one chromogenic substrate) NF validation compared to ISO 21527	**3M Petrifilm Lactic Acid Bacteria (LAB) Count Plate (sample-ready-culture-medium system containing one chromogenic substrate)** NF Validation compared to ISO 15214	**3M Petrifilm AC/RAC plate (sample-ready-culture-medium system containing one chromogenic substrate)** NF validation (AFNOR) (as compared to ISO 4833-1 method)
AOAC 2014.05 3M RYM AOAC 997.02 3M YM	AOAC 2017	AOAC 990.12 3M AC AOAC 2015.13 3M RAC
AFIA 1996	**AFIA 1996**	**AFIA 1996**
	Compendium Microbiological Examination of Foods Chapter 9/10 (**CMMEF**), Fifth edition 2015	
	SL-01 18 hours O/N shaking at RT)	

Matrixes for 3M-LAB plate, AC plate, and RYM plate
Bakery, Beverage & Bottled Water, Confectionary, Dairy, Eggs, Fruits & Vegetables, Grain & Oilseed Milling Sector, Meat, Nutraceuticals, Pet Food & Animal Feed, Poultry, Prepared & Processed Foods, Seafood

Enumeration Methods of Microorganisms for Animal Feed and Pet Food
Total Lactic Acid Bacteria Count Determination (viable plate count method)

Methods	Media	Pre-treatment	Diluent	Incubation temp.	Incubation condition	Note
AFIA 1996	MRS agar Amphotericin B	Blend or homogenize sample 60-90 sec	0.1% peptone H_2O 0.1% T80	37°C 72 hours	Anaerobic overlay agar medium	
ISO 15214 (1998)	MRS agar, pH 5.7	Blend or homogenize sample	0.1% peptone saline	30°C 72 hours	Anaerobic overlay agar medium	ISO 16140-Method validation AOAC Europe
3M LAB Petrifilm (AOAC 2017)	Selective nutrients Anti-fungi agent tetrazolium indicator	Blend or homogenize sample	0.1% peptone H_2O (Buffer) or Buffered phosphate buffer	28°C to 37°C 48 hours ± 3 hours	Anaerobic oxygen scavenging compounds	AOAC Performance Tested Method Certificate #041701 (only certificated LAB method) NF VALIDATION certified method in compliance with ISO 16140-28 in comparison to ISO 15214
CMMEF	MRS agar (modified)	Blend or homogenize sample	0.1% peptone H_2O Stress or damaged in MRS broth	37°C 48 to 72 hours	Anaerobic overlay agar medium or anaerobic chamber w/gasPak	Compendium of Method for Microbiological Examination of Food Chapter 19/20
SL-01	MRS agar	18 hrs RT shaking at 200rpm	Water or Butterfileld's phosphate/0.1% T80	37°C 72 hours 43°C 72 hours	Anaerobic	

Enumeration Methods of Microorganisms for Animal Feed and Pet Food
Total Microbial Count Determination

AFIA 1996

AMERICAN FEED INDUSTRY ASSOCIATION

October 8, 1996

TO: AAFCO State Contacts,

The AFIA Microbial, Enzyme, and Forage Technology Council (MEFT) appointed a task force to review the analytical methods for direct-fed products. This task force has completed their review and as a result, the MEFT Council is publishing three methods, Enumeration of Bacillus, Enumeration of Yeast, and Enumeration of Lactic Acid Bacteria.

The Council has adopted these methods and recommended that they be made available to regulators and testing laboratories.

These methods represent a consensus of methods employed by members of the MEFT Council. They have been agreed to as reasonable for use in enumerating products which contain viable microorganisms. As with most consensus methodologies, they may not be completely acceptable for every viable product in the market. If difficulties are encountered in the use of these methods with a particular sample, it is suggested the manufacturer of the product in question be contacted. The completion of this project does not mean that all companies will use these methods as in-house methodology. It only means that the members of the Council are satisfied that the methods are acceptable for use by regulatory official, customers, or other interested labs.

If you have any questions concerning these methods, please do not hesitate to contact me.

Sincerely,

Betty Pendleton

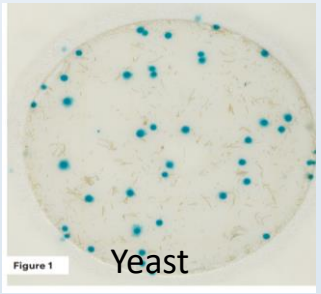
Enumeration Methods of Microorganisms for Animal Feed and Pet Food
Total Microbial Count Determination

AFIA 1996	Enumeration of Bacillus	Enumeration of Yeast/Mold	Enumeration of Lactic Acid Bacteria
Medium	Tryptic Soy Agar (TSA)	Potato Dextrose Agar (PDA) Tartaric Acid 10% solution /CTC	MRS medium Amphotericin B
Extraction/dilution buffer	10g sample+90ml 0.1% peptone/0.1% Tween 80	10g sample+90ml 0.1% peptone/0.1% Tween 80	10g sample+90ml 0.1% peptone/0.1% Tween 80
Blend	Low speed for 1.0 to 1.5 min	Low speed for 1.0 to 1.5 min	Low speed for 1.0 to 1.5 min
Recovery	Shaking for 25-30 times	Shaking for 25-30 times	Shaking for 25-30 times
Sample dilution	10-fold dilution	10-fold dilution	10-fold dilution
Heat treatment	10 min 80°C in water bath/cool in RT		
Plating	Add 1ml diluted microbial sample to petri dish, 14 ml of 45°C TSA agar. Gentle mix in one direction and then the other direction. Optional: overlay 7 ml after the bottom layer is solid.	Add 1ml of diluted sample to petri dish and 14 ml of 45°C PDA with 1 ml 10% Tartaric Acid per 100 medium.	Add 1ml of diluted sample to petri dish and 14 ml of 45°C MRS agar with amphotericin B. Mix gently in one direction and then other direction. Overlay 7 ml after the bottom layer solid to create anaerobic environment.
	37°C 48-72 hours	25°C 3-5 days	37°C 72 hours

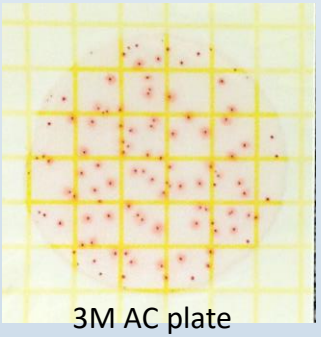
Enumeration Methods of Microorganisms for Animal Feed and Pet food
Total Microbial Count Determination with 3M Petrifilm

Mold

3M RYM



Yeast



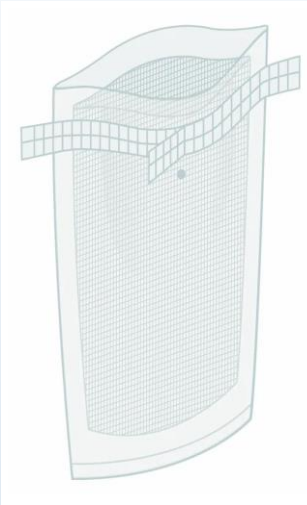
3M AC plate

10 grams feed sample + 90 ml 0.1% peptone/0.1% T80

Blending for 90 sec → filter bag

↓

10-fold dilutions

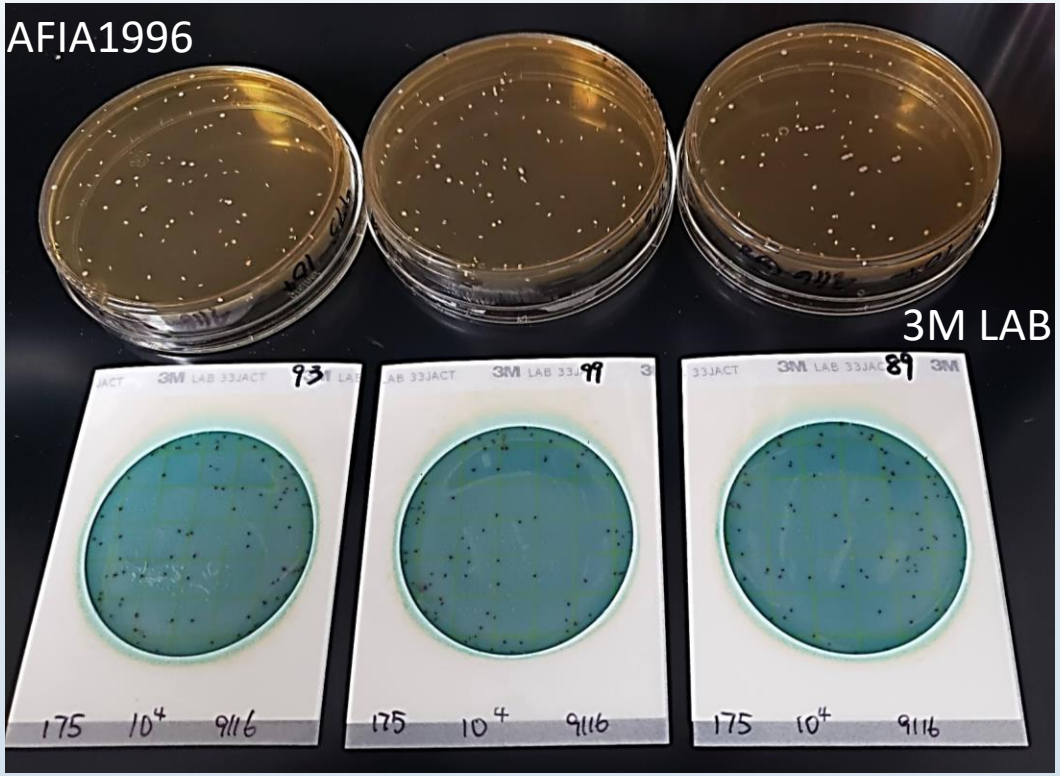


Bacillus (80°C 10min)	+ Lactic Acid Bacteria	+ Yeast/Mold
↓	↓	↓
1ml on 3M petriflm AC/RAC plate	1ml on 3M petrifilm LAB plate	1ml on petrifilm RYM/YM plate
↓	↓	↓
Incubate at 30-37°C 24-48 hrs	Incubate at 37°C 72 hrs	Incubate at 25-28°C 48-72 hrs (5days for YM)

Comparison of *Bacillus* counts (AFIA 1996) with **3M** AC petrifilm counts

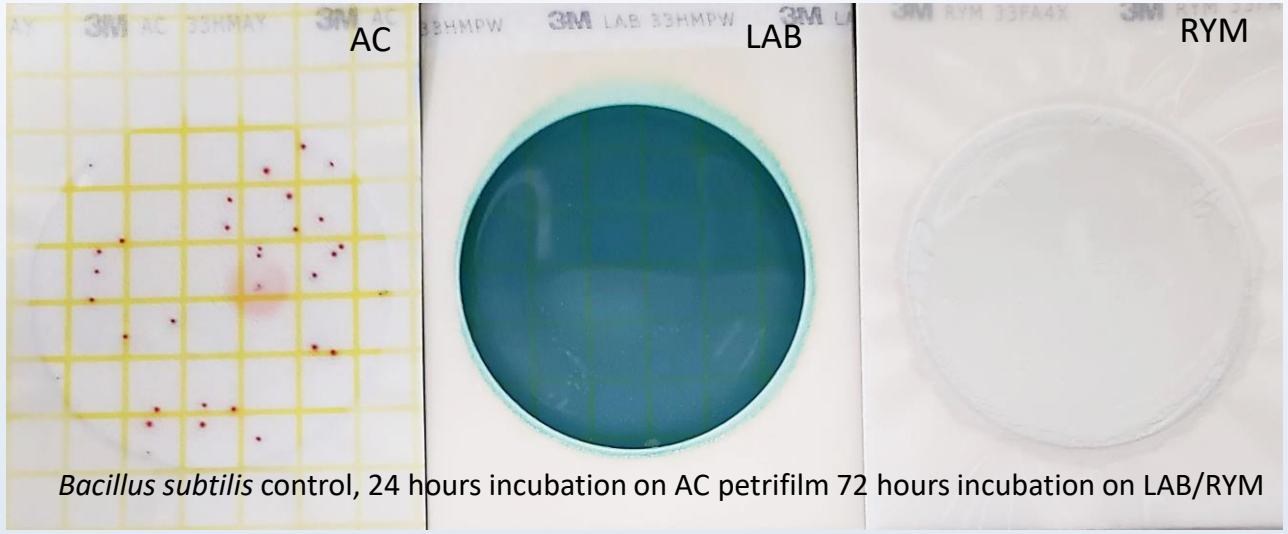
	Feed sample	Rep1 (CFU)	Rep2 (CFU)	Rep3 CFU)	Ave (CFU)	Ave(TSA/AC)	SD	CV%
Run 1	TSA plate (AFIA1996)	167980000000	199760000000	181600000000	183113333333			
	3M AC petrifilm	177060000000	195220000000	217920000000	196733333333	189923333333	9630794360	5.0709
Run 2	TSA plate (AFIA1996)	131660000000	167980000000	213540000000	171060000000			
	3M AC petrifilm	158900000000	190680000000	183110000000	177563333333	174311666667	4598551100	2.6381
Run 3	TSA plate (AFIA1996)	149820000000	181600000000	177060000000	169493333333			
	3M AC petrifilm	158900000000	190680000000	199760000000	183113333333	176303333333	9630794360	5.4626
Run 4	TSA plate (AFIA1996)	208840000000	208840000000	222460000000	213380000000			
	3M AC petrifilm	195220000000	190680000000	213380000000	199760000000	206570000000	9630794360	4.6622
	All 4 runs					186777000000	2516121630	1.3471

Comparison of *Lactic acid bacteria* counts (AFIA 1996) with **3M** LAB petrifilm counts

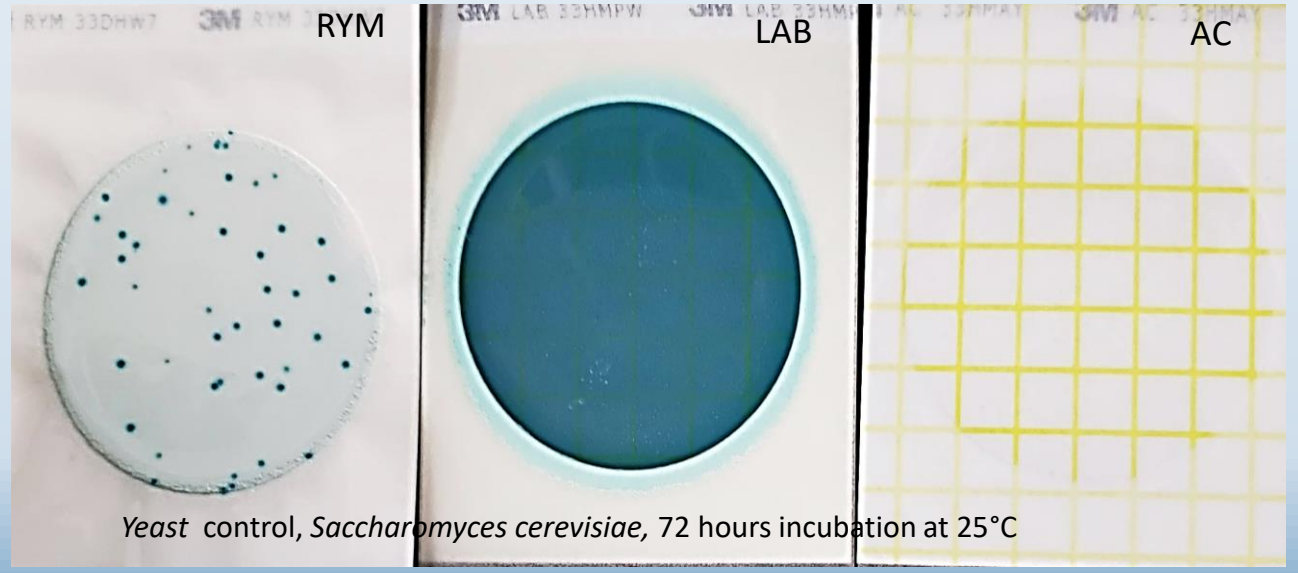


Sample	Assay	rep1 (CFU)	rep2 (CFU)	rep3 (CFU)	Average (CFU)	SD	RSD%
Run 1	LAB petrifilm	630000	480000	700000	**603333**	112398.10	18.63
	MRS plate (AFIA1996)	490000	540000	600000	**543333**	55075.71	10.14
					573333	42426.41	7.40
Run 2	LAB petrifilm	1260000	1260000	1040000	**1186667**	127017.06	10.70
	MRS plate (AFIA1996)	1410000	1650000	1920000	**1660000**	255147.02	15.37
					1423333	334697.21	23.52
Run 3	LAB petrifilm	1160000	1220000	1350000	**1243333**	97125.35	7.81
	MRS plate (AFIA1996)	1860000	1320000	1360000	**1513333**	300887.58	19.88
					1378333	144081.65	10.45
All 3 runs					1125000	148374.70	13.19

Growth Specification on 3M petrifilms with control strains from ATCC or NRRL (Agriculture Research Service Culture Collection)

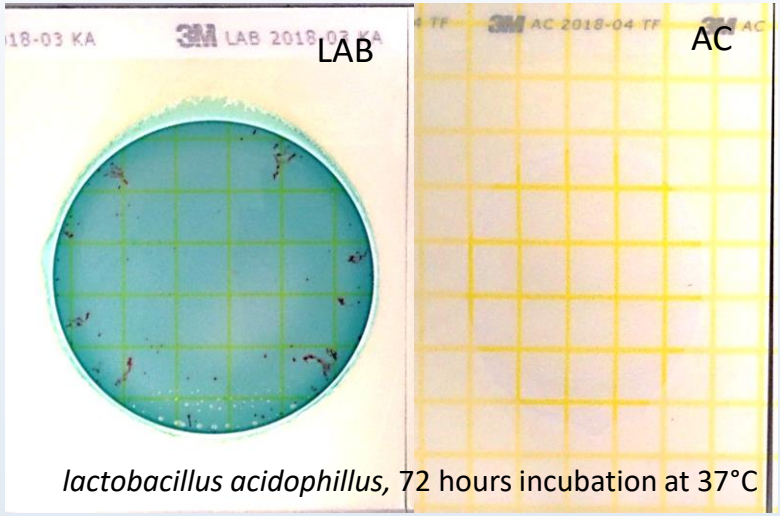


Bacillus subtilis control, 24 hours incubation on AC petrifilm 72 hours incubation on LAB/RYM

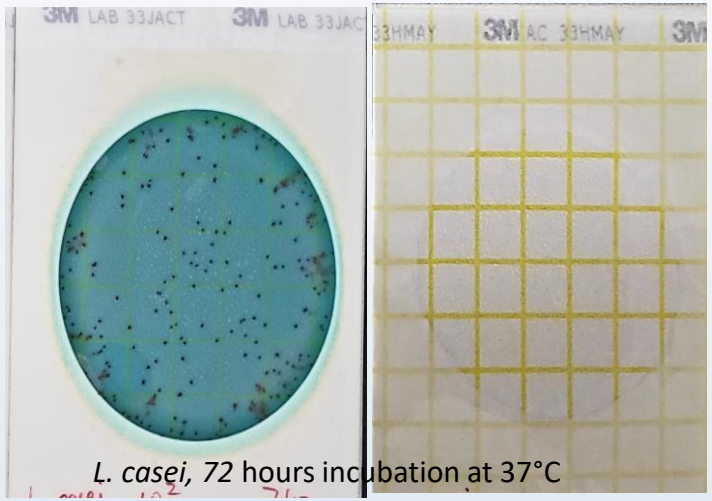


Yeast control, *Saccharomyces cerevisiae,* 72 hours incubation at 25°C

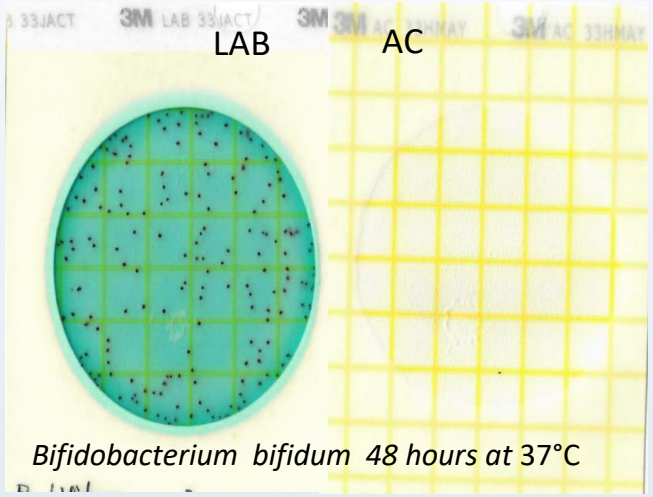
Growth Specification on 3M petrifilms with control strains from ATCC or NRRL (Agriculture Research Service Culture Collection)



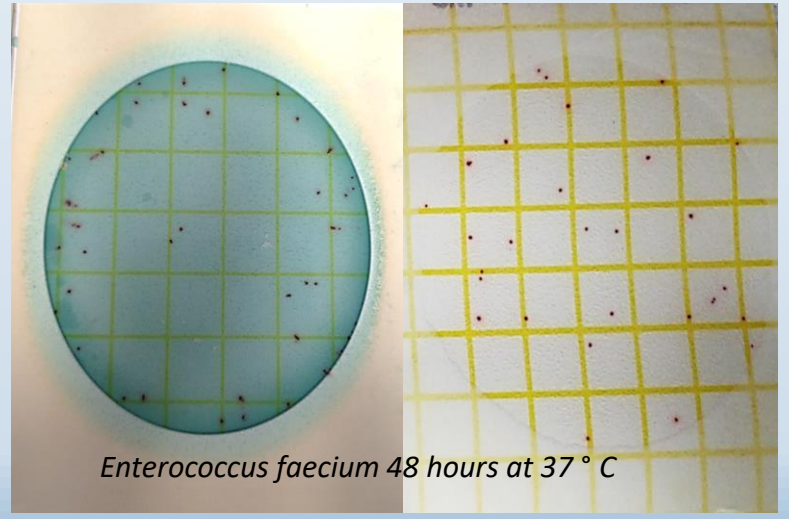
lactobacillus acidophillus, 72 hours incubation at 37°C



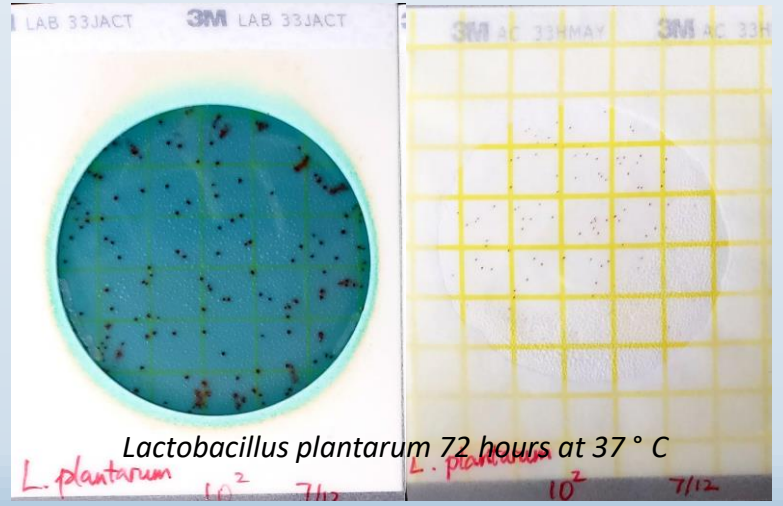
L. casei, 72 hours incubation at 37°C



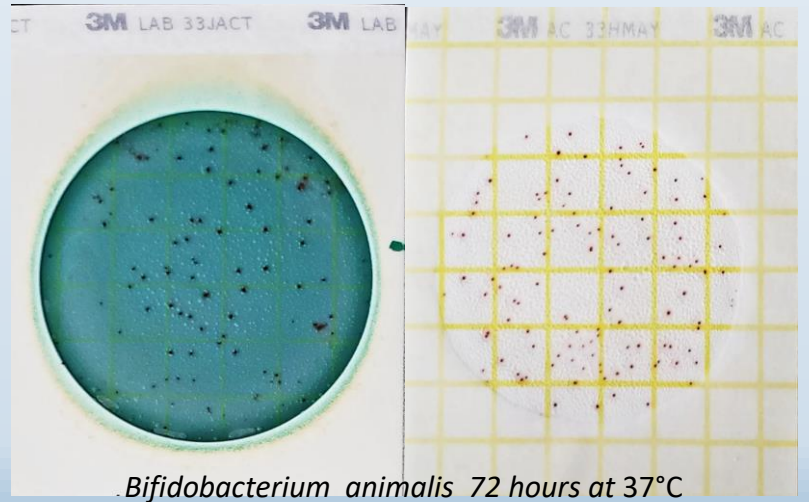
Bifidobacterium bifidum 48 hours at 37°C



Enterococcus faecium 48 hours at 37 ° C

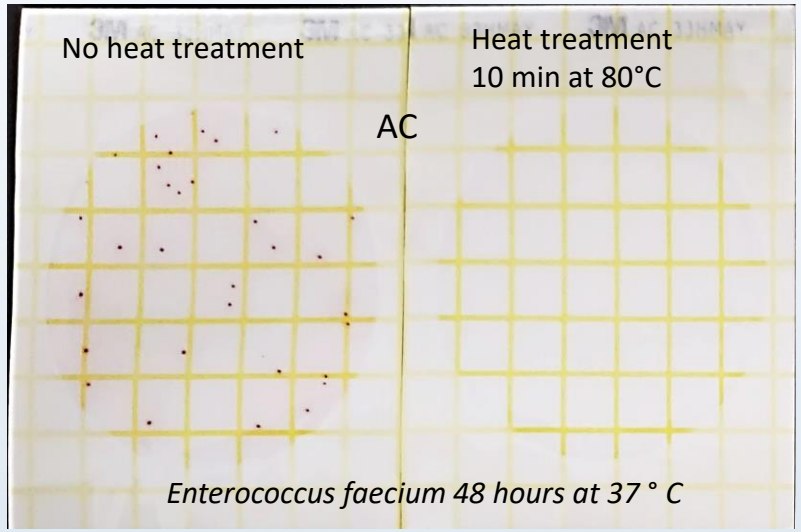


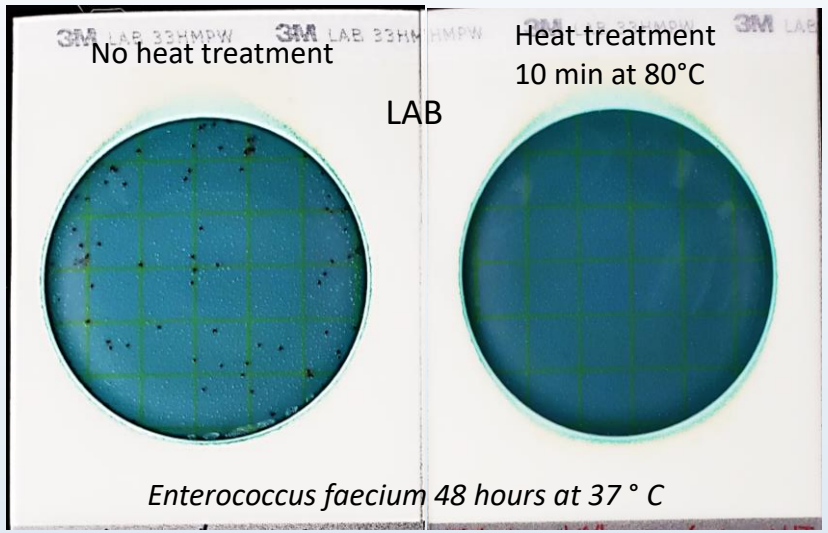
Lactobacillus plantarum 72 hours at 37 ° C



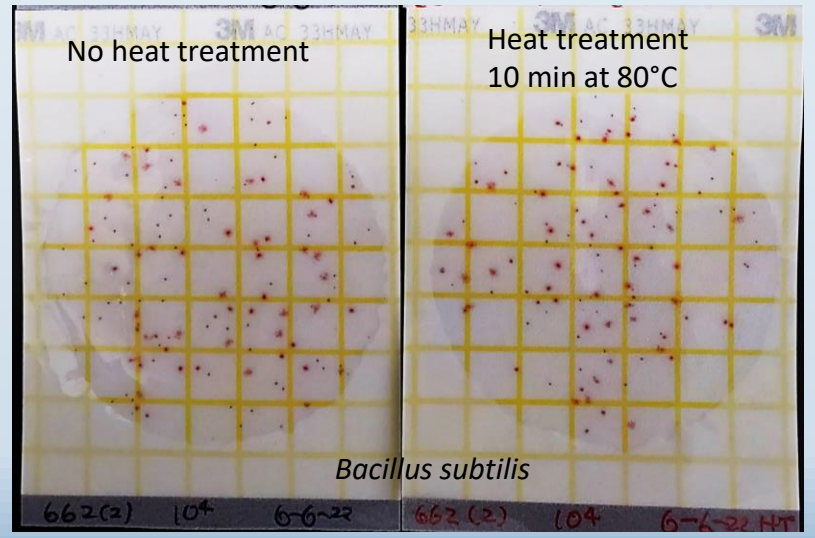
Bifidobacterium animalis 72 hours at 37°C

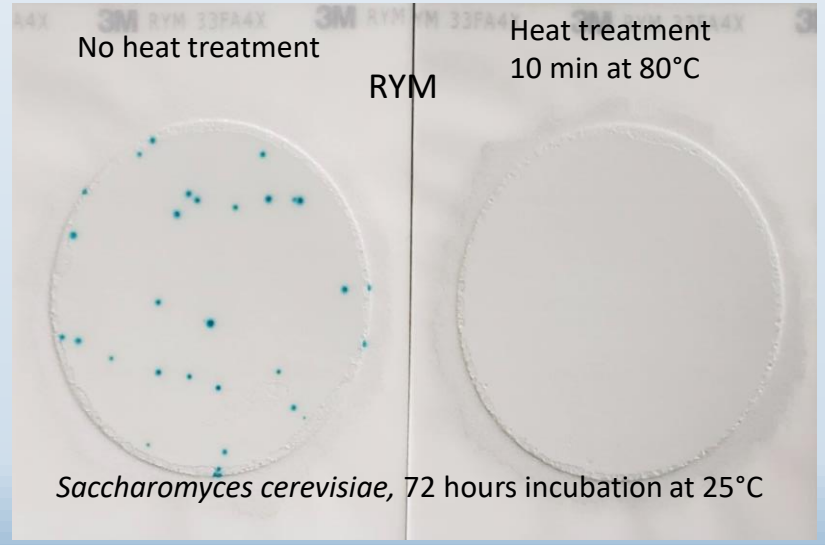
Growth Specification on 3M petrifilms with control strains from ATCC or NRRL (Agriculture Research Service Culture Collection)





80°C 10 min heat treatment efficiently killed Lactic acid Bacteria





80°C 10 min heat treatment didn't change Bacillus count

Summary

- No significant difference of microorganism counts between AFIA 1996 plate method and 3M petrifilm LAB (AOAC 2017) method.

- In an AOAC RI PTM study, the 3M Petrifilm LAB Count Plate method was found to be equivalent to the average log counts of Compendium of Methods for the Microbiological Examination of Foods (**CMMEF**) Chapter 19, Fifth Edition and the **ISO 15214**: Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria –colony-count technique at 30°C, First edition, 1998-08-01.

http://www.keydiagnostics.com.au/images/PDF/MMM/3M_Petrifilm_Lactic_Acid___-_Instructions_KD_06-18.pdf

3M LAB petriflim plate, ISO 15214, CMMEF, and AFIA 1996 methods are all similar for LAB counts.

- SL-01 is most different than other above methods. Method doesn't have blend sample step and has an 18 hours 200 rpm overnight shaking at RT. *Increase some species counts

Sample collection and processing

Grinding and no grinding

	Sample
90 sec Blending in diluent	5775
No Blending	2600

30 min resuscitate step might be necessary for Recovery of cells from dried cultures and products

All results present in this presentation used 3M petrifilm plate counts method in the next few slides

Inspectors collect microbial samples in winter season

↓

Grinding room
500 grams sample from original bag into sterile jar save in fridge

↓ In cooler

Microbiology Lab (samples save in fridge)
10 grams+90 ml diluent (0.1% pep/0.1% T80)
Blending in sterile blender
two runs and 3 reps each run with positive and negative controls

↓

Report results out
If sample is homogeneous

40% low AV limit and
No up AV limit

↓

Grinding whole jar of the sample if not homogeneous

↓

Run sample again

Labeled direct-fed microorganisms with separate guaranteed analysis for different groups of microorganisms

Total Lactic Acid Microorganisms , minimum ……..1,000,000 CFU/lb (*Lactobacillus acidophilus*, *Bifidobacterium longum, Enterococcus faecium*)
Total Bacillus microorganisms, minimum…… 10,000,000 CFU/lb (*Bacillus subtilis, Bacillus licheniformis*)
Total yeast microorganisms, minimum……500,000 CFU/lb (*Saccharomyces, cerevisiae*)

ID	Species	Guarantee (min)	Results	P/F	Note
Sample 1	Total Bacillus 2	7,000,000 CFU/lb	14,881,273	Pass	> Easy to see which groups of microorganisms failed guarantee analysis > Easy to point any problem of microorganisms counts in products. > Stability, viability, and other issues.
	Total LAB 5	100,000,000 CFU/lb	7,968,555	low	
Sample 2	Total Bacillus 2	150,000,000 CFU/lb	235,323,334	Pass	
	Total LAB 4	750,000,000 CFU/lb	22,649,556	low	
Sample 3	Total Bacillus 1	38,000 CFU/ml	320,000	Pass	
	Total LAB 2	1,300,000 CFU/ml	343,300	low	
	Total yeast 2	3,530,000 CFU/ml	5,000,000	Pass	
Sample 4	Total Bacillus 1	2000000000 CFU/kg	1,450,000,000	Pass_low	
	Total LAB 4	12000000000 CFU/kg	116,666	low	
	Total Yeast 1	315,000,000,000 CFU/kg	36,500,000,768	low	
Sample 5	Total Bacillus 2	30 million CFU/lb	55.94	Pass	
	Total yeast 1	450 million CFU/lb	174.09	Low	

Bacillus licheniformis, Bacillus subtilis

Saccharomyces cerevisiae

Labeled direct-fed microorganisms with one total guarantee analysis CFU/g or CFU/lb for cross different groups of microorganisms

Total Microorganisms (*Lactobacillus acidophilus, Enterococcus faecium, Bacillus subtilis*) Min……40 Million CFU/lb

ID	Species	Guarantee (min CFU/g)	Results (CFU/g)	Total (CFU/g)	P/F	Note
Sample 1	Bacillus 1	176,200	905,333	1,005,533	pass	* One group of microorganisms could be high enough to cover the failed group of microorganisms (sample 5). *One group of microorganisims could be low enough to fail the guaranteed analysis (sample 4).
	LAB 4		100,200			
Sample 2	Bacillus 1	176,211	384,333	355,990	pass	
	LAB 4		28,343			
Sample 3	Bacillus 2	1,145,374	73,183	147,293	low	
	LAB 3		4,110			
	Yest 1		70,000			
Sample 4	LAB 4	30,000	4,167	27,067	low	
	Mold 1		22,900			
Sample 5	LAB 4	50,000	711	70,818	pass	
	Asp.(Mold) 1		70,107			

Labeled direct-fed microorganisms as total CFU/g or CFU/lb with a single family of microorganisms

Total microbial count, minimum …….. 22000000 CFU/LB (*Bacillus subtilis*, *Bacillus licheniformis*)

ID	Species	Guarantee (min)	P/F	Note
sample 1	Total *Bacillus*	200,000,000 CFU/g	pass	* Bacillus species are known for their ability to form spores. * Bacillus group of bacteria are very stable in harsh environment. Sporulated Bacillus strains will remain stable and viable for long time.
sample 2	Total *Bacillus*	84,000,000 CFU/lb	pass	
sample 3	Total *Bacillus*	100,000,000 CFU/lb	pass	
smaple 4	Total *Bacillus*	80,000,000 CFU/lb	pass	
sample 5	Total *Bacillus*	84,000,000 CFU/lb	pass	
sample 6	*B. coagulans*	80,000,000 CFU/lb	low	
sample 7	*B. coagulans*	7,800,000 CFU/lb	pass	
sample 8	*B. coagulans*	7,800,000 CFU/lb	pass	
sample 9	*B. coagulans*	17,900,000 CFU/g	pass	
sample 10	total *Bacillus*	200000000 CFU/g	pass	
sample 11	total *Bacillus*	84,000,000 CFU/lb	pass	

ID	Species	Guarabtee (min)	P/F	Company
sample 1	Saccharomyces cerevisiae	12,000,000 CFU/lb	Pass	GRO-TEC
sample 2	Saccharomyces cerevisiae	315,000,000,000 CFU/kg	Fail	
sample 3	Saccharomyces cerevisiae	2,400,000 CFU/lb	Pass	GRO-TEC
sample 4	Saccharomyces cerevisiae	12,000,000 CFU/lb	Pass	GRO-TEC
sample 5	Saccharomyces cerevisiae	30,000,000 CFU/lb	Pass	Scott Pet INC

Labeled direct-fed microorganisms as total CFU/g or CFU/lb with a single family of microorganisms

ID	Assay	Species	Guarantee (min CFU/lb)	Results (CFU/lb)	P/F	Company	Collect date	Best By	lot#	location	Product name
Sample 1	Total LAB	*L. plantarum* *E. faecium* *L. casei* *L. acidophilus*	20,000,000	656,030,000	Pass	XXX	2/23/2022	Aug-23	0523 P2 L5 MO 0492	MISHAWAKA, IN	Wellness Complete Health Small Breed Healthy Weight Dog Food
Sample 2			80,000,000	34,857,111	Low	XXX	2/23/2022	Aug-23	2004 P2 L4 MO 0532	MISHAWAKA, IN	Wellness CORE small breed Grain free Protein-rich Healthy weight Dog Food
Sample 3			80,000,000	8,081,200	Low	XXX	2/23/2022	Jul-23	0102 P2 L4 MO 0322	MISHAWAKA, IN	Wellness CORE large breed Grain free Protein-rich for Puppy
Sample 4			90,000,000	353,363,333	Pass	XXX	2/23/2022	Aug-23	1658 P2 L4 MO 0522	MISHAWAKA, IN	Wellness Core Grain Free Protein Rich Nutrition Indoor Cat food
Sample 5			90,000,000	57,506,667	Pass	XXX	2/23/2022	Apr-23	1450 P2 L2 MO 3031	INDIANAPOLIS, IN	Wellness Core Grain Free Protein Rich Nutrition Indoor Cat food

This company's dry pet foods have an expiration of 18 months from date of manufacture

Lactic Acid Bacteria, total – MI 302 (Based on CMMEF, Microbiological Examination of Food, Chapter 19)

A representative sample is obtained and combines with phosphate buffer. Aliquots of the sample are placed on Sterile petri dishes, 15-20 ml of MRS agar is added, swirled, and solidified. The plates are incubated for 48 to 72 hours in anaerobic and aerobic condition.

Labeled direct-fed microorganisms as total CFU/g or CFU/lb with a single family of microorganisms

ID	Assay	species	Guarantee	Results	P/F	Company	Collect date	lot#	Collect location	Product name
22-0024	Total LAB	*E. faecium* *L.acidophilus* *L. casei*	200,000 (CFU/g)	1,193	low	[redacted]	2/1/2022	L 21 1791 B 12/28/2022	KOKOMO, IN	[redacted] Sunburst Gourmet Blend with protein egg food for parakeet
22-0707		*L. acidophilus* *L. casei* *L. plantarum*	220,000 (CFU/g)	933	low		3/21/2022		FORT WAYNE, IN	[redacted] Sustain Premium Recipe with Wild Caught Alaskan Salmon Puppy Dog food
22-0033		*L. acodophius* *B. animalis* *L. casei*	100,000,000 (CFU/lb)	35,008,444	low		2/10/2022 2 yrs	3023118-12255 BB 12 FEB 2023 1	SOUTH BEND, IN	[redacted] First Feast with Free Run Chicken & Whole Herring Kitten Food
22-0275		*L. casei* *L. acidophilus* *B. animal* *L. reuteri*	10,000,000 (CFU/lb)	1,961,280	low		1/5/2022	EXP 03/23 LOT/82138	CLARKSVILLE, IN	[redacted] Hedgehog Essential Hedgehog Food with Whole Dried Insects
22-0857		*L. acidophilus* *L. plantarum* *L. reuteri* *B. animalis*	1,000,000 (CFU/Lb)	246,673,333	pass		3/16/2022	TD208/FERUARY /2022 2209 Best If use by 02/08/2023	Aurora, IN	[redacted] Pure Being Deboned Salmon, Rice, & Sweet Potato Recipe Cat Food
22-1320		*L. acidophilus* *E. faecium*	90,000,000 (CFU/Lb)	582,633	low		7/7/2022	BB 2023 MR 02 11101 52300 CDO3	INDIANAPOLIS, IN	Go! Skin+Coat Care Chicken Recipe with Grains Dog Food
22-1525		*B. thermophilum* *E. faecium* *L. casei* *L. acidophilus*	264,000,000 (CFU/lb)	2,595,367	Low		8/3/2022	348375BF	GOSHEN, IN	Premium Mixing Pellet 36 80026AAA

Labeled direct-fed microorganisms as total CFU/g or CFU/lb with a single family of microorganisms

ID	Assay	species	Guarantee (min CFU/lb)	Results (CFU/lb)	P/F	company	Collect date	lot#	Collect location	Product name
Sample 1	Total LAB	*L. acidophilus* *L. casei* *B. thermophilum* *E. faecium,*	20,400,000	1,944,633	low	Company A	2/28/2022	6020100131	MARION, IN	NATUREWISE LAYER FEED CRUMBLE (ME)
Sample 2			20,400,000	4,494,600	low		2/28/2022	6012510106	MARION, IN	NATUREWISE LAYER FEED PELLET (ME)
Sample 3			10,200,000	2,220,060	low		2/28/2022	6020210326	MARION, IN	NATUREWISE CHICK STARTER/GROWER 18% CRUMBLES
Sample 3			20,400,000	1,638,940	low		1/26/2022	BY13501731 16DEC21	MARION, IN	PROFORCE Senior (BY) Senoir maintenance and performance horses
Sample 4			20,400,000	1,225,800	low		3/25/2022	6020310431 31JAN22	ELKHART, IN	Safechoice Perform pellet (ME)
Sample 5			20,400,000	2,326,293	low		2/23/2022	6012170073	MARION, IN	NATUREWISE LAYER FEED PELLET (ME)
Sample 6			20,400,000	696,133	low		2/21/2022	6013140155	NAPPANEE, IN	SafeChoice Original Horse Feed (ME)
Sample 7			20,400,000	1,638,940	Low		1/26/2022	BY13501731 16DEC21	LINTON, IN	PROFORCE Senior (BY) Senoir maintenance and performance horses
Sample 8			20,400,000	2,248,813	Low		1/4/2022	2513053476 01NOV21	TELL CITY, IN	Naturewise Meatbird Complete 22% Crumble

SL-01 method:
Lactic Acid Bacteria Counting Procedure for Feed Products containing PrimaLac FG, PrimaLac Water Soluble.

SL-01 method for LAB enumeration

Weigh approximately 10.0g or (50.0g for feeds)
into sterile dilution bottle
(refer to sample weighing section for details)

↓

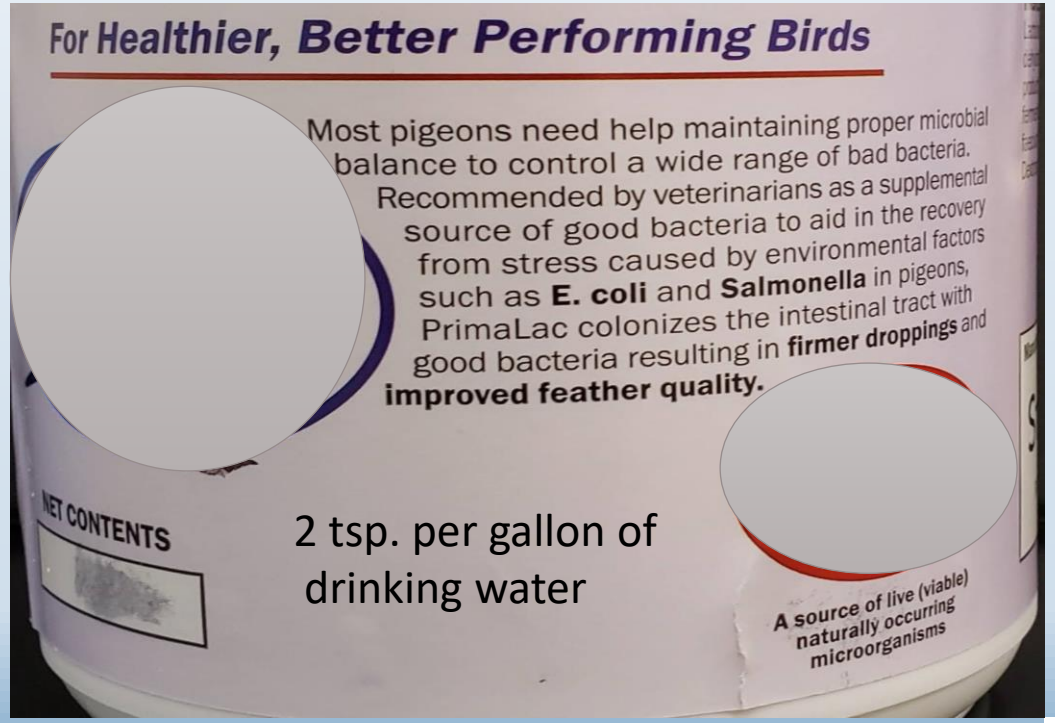
Mix 18 hours at room temperature at a low to
Moderate speed. Do not exceed 18 hours.

AVG of 3 reps	L.acidophilus	L.rhamnosus	B.thermophilum	E.faecium
10C Cold	2.49E+04	1.04E+03	1.99E+04	2.14E+04
37C Hot	1.23E+06	3.96E+05	1.23E+06	1.66E+05
23C RT (SL-01)	2.42E+04	1.65E+03	1.88E+04	2.05E+04

No shaking control?

INGREDIENTS:
Lactobacillus acidophilus fermentation product dehydrated, Lactobacillus casei fermentation product dehydrated, Bifidobacterium thermophilum fermentation product dehydrated, Enterococcus faecium fermentation product dehydrated, Dextrose and Citric acid.

L. acidophilus, L. casei, B. thermophilum, E. faecium



Overnight shaking Vs no overnight shaking with control LAB strains (ATCC or NRRL) and feed samples

Ratio	Species	RT/no-shaking control	4°C O/N shaking	RT O/N Shaking
control	*L. acidophilus*	1	1.06	1.26
		1	0.78	1.27
		1	0.91	1.26
		1	1.41	2.93
	B. bifidum	1	1.24	22.97
		1	1.24	10.51
	L. casei	1	0.86	3.27
		1	0.98	TNTC
	E. faecium	1	0.91	671.88

Ratio	Feed sample	RT/no-shaking control	4°C O/N shaking	RT O/N Shaking
Sample 1	*Bifidobacterium thermophi*	1	1.02	37.74
	Enterococcus faecium			
	Lactobacillus casei			
	Lactobacillus acidophilus			
Sample 2	*Lactobacillus plantarum*	1	0.86	9.86
	Enterococcus faecium			
	Lactobacillus acidophilus			
	Lactobacillus reuteri			
	Bifidobacterium animalis			
Sample 3	*Bifidobacterium thermophi*	1	0.8	TNTC
	Enterococcus faecium			
	Lactobacillus casei			
	Lactobacillus acidophilus			

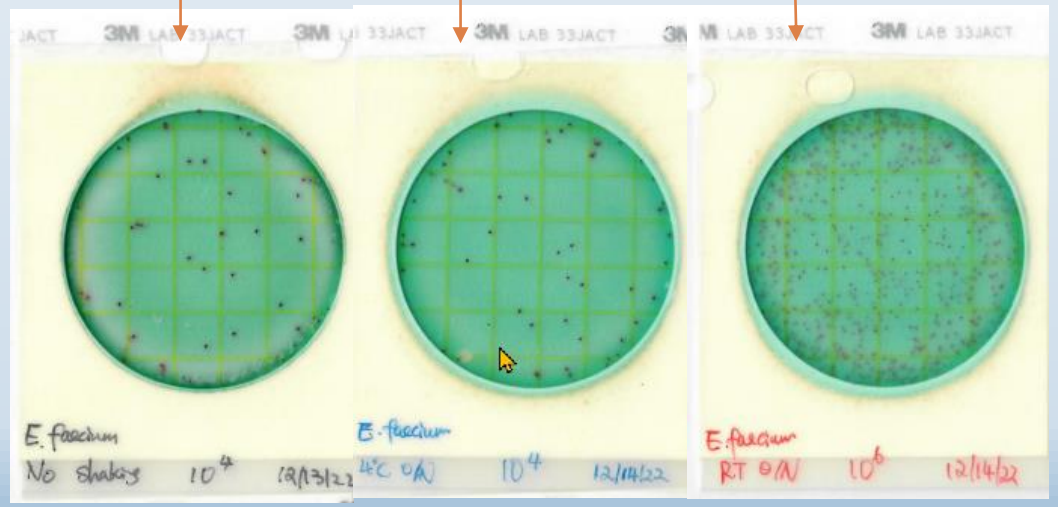
Inoculate 1 ml control strain (estimate concentration 10^8 CFU/ml) to 10-gram sterilized cat food or weight 10-gram of sample

90 ml diluents, blending 90 sec.

Dilution plating

4°C 18 hours shaking Dilution plating

RT ≤18 hours shaking Dilution plating



Sensitivity to heat for control *L. acidophilus* and a LAB cat food sample

Control	Media	No heat treatment (NHT)	Heat treatment sample in water bath	% decrease compared to control NHT
		CFU/ml	**56°C 60min**	
L. acidophilus	MRS plate	7000000000	20000	99.9997
		5800000000	20000	99.9997
			60°C 30min	
L. acidophilus		6900000000	520000	99.9925
		6600000000	470000	99.9929
Feed sample		No heat treatment (NHT)		% decrease compared to control NHT
		CFU/g	**56°C 60min**	
Eagle Kitten	MRS plate	1200000	960000	20.0000
		1070000	850000	20.5607
			60°C 120min	
Eagle Kitten		720000	360000	50.0000
		670000	290000	56.7164
			65°C 120min	
Eagle Kitten		720000	42000	94.1667
		670000	49000	92.6866

It has been found by several researchers that an increase in fat content in the heating substrate leads to a higher bacterial heat resistance, which may be due to a decrease in water activity

https://www.researchgate.net/publication/229713899_The_protective_effect_of_fat_on_the_heat_resistance_of_bacteria_I

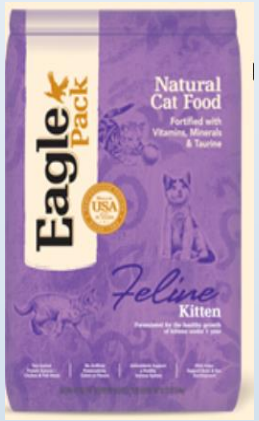
GUARANTEED ANALYSIS

Crude Protein	(min.)	34.00%
Crude Fat	(min.)	21.00%
Crude Fiber	(max.)	3.80%
Moisture	(max.)	10.00%
Calcium	(min.)	0.95%
Phosphorus	(min.)	0.80%
Vitamin A	(min.)	22,000 IU/kg
Vitamin D	(min.)	1,200 IU/kg
Vitamin E	(min.)	75 IU/kg
Taurine	(min.)	0.17%
Ascorbic Acid (Vitamin C)	(min.)	30 mg/kg
Omega 6 Fatty Acids	(min.)	3.80%
Omega 3 Fatty Acids	(min.)	0.51%
Total Lactic Acid Micro-organisms*	(min.)	100,000,000 CFU/lb

(Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium in equal amounts)

*Not recognized as an essential nutrient by the AAFCO Cat Food Nutrient Profiles

Eagle Pack Kitten Food is formulated to meet the nutritional levels established by the Association of American Feed Control Officials (AAFCO) Cat Food Nutrient Profiles for growth and gestation/lactation.



Summary What we learned from human probiotic research

- It is the nature of non spore forming lactic acid bacteria, common strains, include *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, are very sensitive to heart and other Elements.

- The probiotic lactic acid bacteria show poor survival in such ways and often do not reach the human gut alive to give the best benefits.

- Many factors affect probiotics' survival and efficacy, Including:
  - Humidity
  - Temperature
  - pH of the environment
  - Packaging
  - Type of strain
  - Life stage of probiotics
  - Other ingredients in the product

- One method to extend shelf-life of sensitive probiotic bacteria is to freeze-dry them. This essentially puts them into a dormant state and can help prolong shelf life. Many probiotic bacteria in capsules are freeze-dried. Ideal conditions for probiotics are cool temperatures and less than 20% relative humidity. As humidity increases, most probiotics begin to quickly lose stability.

Summary **What we leaned from Microbial Count data analysis and testing**

- Fat, protein, and other substances (protectants) may slow down non-spore-forming bacteria from live to dead.
- We see the trend of decreased LAB counts with the older sample from the manufacture BB day. The older the sample the fewer counts of LAB. (New project: Keep samples at RT and test LAB every week).
- LAB are not stable in animal feed and pet food same as observed in the human probiotic.
- **The stability of non-spore forming microorganisms is very important for animal feeds and pet food because products are not supposed to be stored at low temperature (Protect products from moisture).**
- The best buy date on the label is not the microorganism's expiration date. Non-spore-forming microorganisms shelf life is much short than other ingredients. Typical dry dog food has 10 to 12 percent moisture content in a sealed bag. When bag opened and exposure to the air. The moisture will change.

Suggestions and Discussion

- **Label guarantee analysis separately for different groups of microorganisms will help the regulatory office to find which groups of microorganisms have failed the label guarantees.**

- Increase the accuracy of identifying the viability of microorganisms in animal feed and pet food.

- LAB is a major group of bacteria that used in animal feed and pet food. A true label of microorganisms will protect consumers.

- Feedback to the company to identify which group of the microorganisms has stability or other issues in animal feed and pet food products.

- Microorganisms have different shelf life and functionalities.
  * LAB have been widely described for their capability to enhance the animal immune system, helping protect from pathogen.
  * LAB may promote gut health and boost nutrient absorption.
  * Bacillus probiotics, an alternative to antibiotic for livestock production.

Trish Dunn
Feed Administrator

Thank you

OISC Microbiology laboratory staff
Ju Sheng, Mark Moelhman, Min Chen

Katie Simpson
Pet Food Specialist