Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road
Woodbridge, VA 22192-575.5
703 590 7337 (Fax 703 580 8637)
Smedley@cfr-services.com

Since 1973
consultants to the regulated industry

March 29, 2024

David Edwards Director
Division of Animal Food Ingredients (HFV- 220)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

Subject: Filing of Animal GRAS Notice
DFM *Ruminococcus bovis ASCUSDY10* -
for Dairy Cattle

Notifier: Native Microbials, Inc.
1155 Island Avenue,
Suite 700
San Diego, CA 92101

Dear Dr. Edwards:

On behalf of Native Microbials, I am providing an Animal General Recognized as Safe Notice for the use of *Ruminococcus bovis ASCUSDY10* as a direct fed microorganism for use in Dairy Cattle. The submission is compliant with 21 CFR 570.210-255. The GRAS conclusion is based on scientific procedures.

Should you have any questions on the filing, please contact me directly.

Sincerely,
Kristi O. Smedley

Digitally signed by Kristi O. Smedley
DN: cn=Kristi O. Smedley, o=Center for Regulatory Services, Inc., ou=President, email=smedley@cfr-services.com, c=US
Date: 2024.03.30 09:53:58 -04'00'

Kristi O. Smedley, Ph.D.
Consultant to Native Microbials, Inc.

Cc: Kevin Korth, Native Microbials, Inc.

ATTACHMENTS:

Letter of Smedley Authorization to Represent
GRAS Notice *Ruminococcus bovis ASCUSDY10* (Narrative Hard Copy, Full Submission on DVD)

native MICROBIALS

David Edwards
Director
Division of Animal Feeds, HFV-220
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

March 12, 2024

Subject: Authorization for Representation—Kristi O. Smedley Ph.D. for correspondence (written and verbal), agreements, meeting requests, and submission(s) for Native Microbials, Inc.

Dear Dr. Edwards:

We are authorizing Kristi Smedley to act on our behalf for submission and representation of Native Microbials, Inc related to our submission of the GRAS notice for *Ruminococcus bovis* ASCUSDY10) for use as a Direct Fed Microbial (Viable Microbe) in Dairy Cattle.

Her contact information:

Kristi Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
703-590-7337
Fax 703-580-8637
Smedley@cfr-services.com

Please contact the undersigned with any questions.

Sincerely,

(b) (6)

RECEIVED DATE
APR 4, 2024

Kevin G Korth
Director, Regulatory Affairs
Native Microbials, Inc.
1155 Island Ave., Ste 700, San Diego, CA 92121

GRAS Notice for *Ruminococcus bovis* ASCUSDY10 for Use as a Direct Fed Microbial in Dairy Cattle

March 27, 2024

Prepared for: Division of Animal Feeds, (HFV-220)
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Submitted by: Native Microbials, Inc.
1155 Island Ave, Ste 700
San Diego, California 92101

GRAS Notice for *Ruminococcus bovis* ASCUSDY10 for Use as a Direct Fed Microbial in Dairy Cattle

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LIST OF ABBREVIATIONS

AAFCO	Association of American Feed Control Officials
ADF	Acid Detergent Fiber
ANI	Average Nucleotide Identity
AOAC	Association of Official Analytical Chemists
BAM	Bacteriological Analytical Manual
BLAST	Basic Local Alignment Search Tool
BUSCO	Benchmarking Universal Single-Copy Orthologs
CFR	Code of Federal Regulations
CFU	Colony Forming Units
cGMP	current Good Manufacturing Practices
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
CVM	Center for Veterinary Medicine
DFM	Direct Fed Microbial
DM	Dry matter
DNA	DeoxyriboNucleic Acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FSMA	Food Safety Modernization Act
GC	Guanine-Cytosine
GRAS	Generally Recognized As Safe
ITS	Internal Transcribed Spacer
MIC	Minimum Inhibitory Concentrations
NCBI	National Center for Biotechnology Information
ND	Not Detected
NDF	Neutral Detergent Fiber
NRRL	Agricultural Research Service Culture Collection
OP	Official Publication
QPS	Qualified Presumption of Safety
RNA	RiboNucleic acid
SD	Standard Deviation
SPC	Spiral Plate Count
TMR	Total Mixed Ration
USC	United States Code
USP	United States Pharmacopoeia

NOMENCLATURE

The notified substance is *Ruminococcus bovis* ASCUSDY10 and is deposited in the NRRL as B-67764. The microbial strain may be encapsulated with hydrogenated glycerides for use in direct fed microbial products for dairy cattle which is referred to as 'fat encapsulated *Ruminococcus bovis* ASCUSDY10'.

The microbial strain *Ruminococcus bovis* ASCUSDY10 is referred to in some appended reports as 'Dairy-10', 'DY10', or JE7A12^T which are the internal research names for *Ruminococcus bovis* ASCUSDY10.

GRAS Notice for *Ruminococcus bovis* ASCUSDY10 for Use as a Direct Fed Microbial in Dairy Cattle

PART 1 – SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, Native Microbials, Inc. hereby informs the U.S. Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for *Ruminococcus bovis* ASCUSDY10.

1.1 Name and Address of Organization

Native Microbials, Inc.
1155 Island Ave, Ste 700
San Diego, CA 92101

1.2 Name of the Notified Substance

The notified substance is *Ruminococcus bovis* ASCUSDY10 (microbial strain). It is manufactured as a freeze-dried milled product which is further standardized and stabilized by encapsulation in fat for use in direct fed microbial products for dairy cattle. The standardized product is referred to as 'fat encapsulated *Ruminococcus bovis* ASCUSDY10' or '*Ruminococcus bovis* ASCUSDY10 encapsulated'. In addition, a number of the appended reports refer to *Ruminococcus bovis* ASCUSDY10 or the fat encapsulated product under the internal research name, Dairy-10 or DY10.

1.3 Intended Conditions of Use

R. bovis ASCUSDY10 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. The intended purpose of supplementation of the microorganism is to support the digestion of various carbohydrates of animal feed within the rumen. The microbial strain will be delivered in the fat encapsulated form to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *R. bovis* ASCUSDY10 may be incorporated into the diet of dairy cattle include as part of the total mixed ration (TMR), as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. It is anticipated that *R. bovis* ASCUSDY10 will be incorporated into feed at a recommended level of $1x10^8$ CFU/cow/day.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), *R. bovis* ASCUSDY10 manufactured by Native Microbials has been concluded to have GRAS status for use as a direct fed microbial in dairy cattle, as described in Part 1.3, on the basis of scientific procedures.

1.5 Premarket Exception Status

Native Microbials hereby informs the U.S. FDA of the view that *R. bovis* ASCUSDY10 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on Native

Microbials' conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 Availability of Information

The data and information that serve as the basis for this GRAS notification will be made available to the U.S. FDA for review and copying upon request during customary business hours at the offices of:

Native Microbials, Inc.
1155 Island Ave, Ste 700
San Diego, CA 92101

Upon request, Native Microbials will supply the U.S. FDA with a complete copy of the data and information either in an electronic format that is accessible for the Agency's evaluation or on paper. Additionally, the genome sequence of *R. bovis* ASCUSDY10 has been deposited in the National Center of Biotechnology Information (NCBI: CP039381).

1.7 Freedom of Information Act, 5 U.S.C. 552

In Native Microbials' view, nearly all data and information presented in Parts 2 through 7 of this notice do not contain any trade secrets, commercial or financial information that is privileged or confidential, and therefore, all data, and information presented herein, except otherwise indicated, are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552. The indicated exceptions are Appendices 10, 11, 15 and 16, and those that are marked CONFIDENTIAL, which are considered to contain proprietary, confidential commercial information.

1.8 Certification

As required in 21 CFR 570.250(c)(2), Native Microbials, Inc. hereby certifies that to the best of their knowledge, all data and information presented in this notice constitutes a complete, representative and balanced submission, which includes all unfavorable as well as favorable information known to Native Microbials and pertinent to the evaluation of the safety and GRAS status of *Ruminococcus bovis* ASCUSDY10.

Signed,

(b) (6)

Mallory Embree, PhD, Chief Science Officer

Date

PART 2 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Source of the Microorganism

Ruminococcus bovis ASCUSDY10 was isolated to axenicity from a healthy, mid-lactation Holstein cow rumen sample. The strain was isolated by Native Microbials (Native Microbials, 1155 Island Ave., Ste 700, San Diego, CA 92101). The isolate was deposited in the NRRL, Agricultural Research Service Culture Collection, and is referenced as NRRL B-67764 (Appendix 1). The microorganism has also been sequenced (Part 2.2.2 and Appendix 003B) and deposited in the National Center for Biotechnology Information (NCBI) database as *Ruminococcus bovis* strain JE7A12 (Appendix 003C).

2.2 Description of the Microorganism

2.2.1 Physical Characteristics

R. bovis ASCUSDY10 is an obligate anaerobe, catalase negative, and oxidase negative bacterium. It Gram stains positive (Figure 2.1) and forms chains of small cocci when cultured in liquid medium (Figure 2.2). When cultured on tryptic soy agar with ferric ammonium citrate (TSA+FAC) medium, it forms small, slightly opaque, off-white, circular colonies with even margins (Figure 2.3).

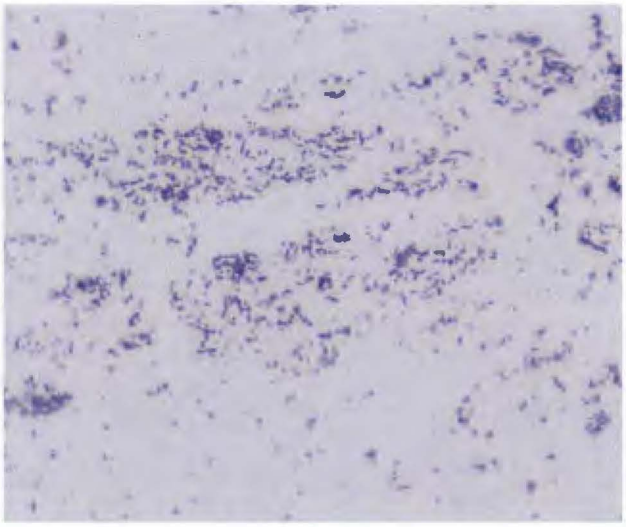

Figure 2.1 R. bovis ASCUSDY10 Gram Stain after 48 hours of incubation (1000x magnification)

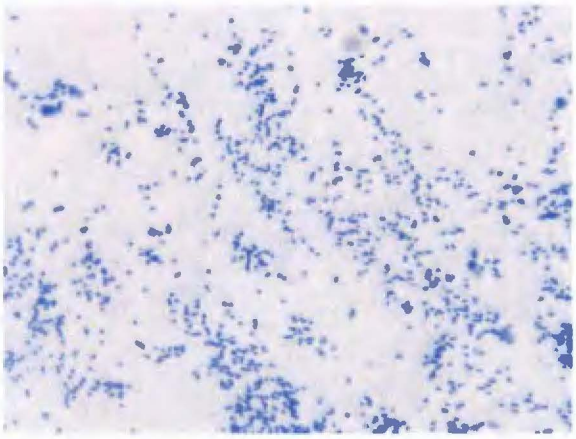

Figure 2.2 R. bovis ASCUSDY10 Methylene Blue Stain after 48 hours of incubation (1000x magnification)

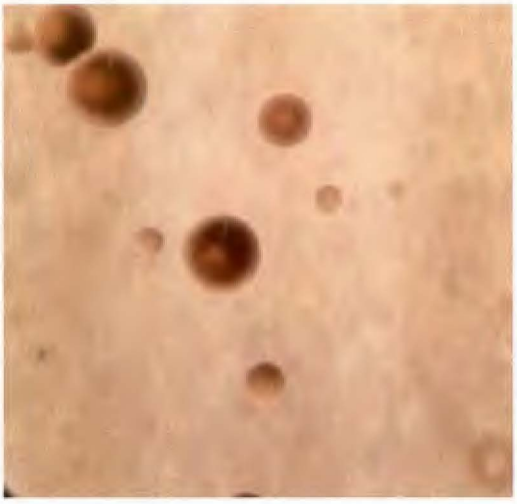

Figure 2.3 R. bovis ASCUSDY10 Colonies on TSA+FAC Agar (4x magnification)

In vitro assays demonstrate that *R. bovis* ASCUSDY10 grows on a variety of mono- and polysaccharides including galactose, glucose, fructose, starch, glycogen, and maltose. Additionally, the species also grows on esculin/ferric citrate. Carbon source utilization results are summarized in Table 2.1.

Table 2.1: Growth of *R. bovis* ASCUSDY10 on Different Carbon Sources			
Carbon Source	**Growth**	**Carbon Source**	**Growth**
No Carbon Control	No Growth	Inositol	No Growth
Glycerol	No Growth	D-Mannitol	No Growth
Erythritol	No Growth	D-Sorbitol	No Growth
D-Arabinose	No Growth	Methyl-aD-Mannopyranoside	No Growth
L-Arabinose	No Growth	Methyl-aD-Glucopyranoside	No Growth
D-Ribose	No Growth	N-AcetylGlucosamine	No Growth
D-Xylose	No Growth	Amygdalin	No Growth
L-Xylose	No Growth	Arbutin	No Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth
Methyl-BD-xylopyranoside	No Growth	Salicin	No Growth
D-Galactose	Growth	D-Cellobiose	No Growth
D-Glucose	Growth	D-Maltose	Growth
D-Fructose	Growth	D-Lactose	No Growth
D-Mannose	No Growth	D-Melibiose	No Growth
L-Sorbose	No Growth	D-Saccharose	No Growth
L-Rhamnose	No Growth	D-Trehalose	No Growth
Dulcitol	No Growth	Inulin	No Growth
D-Melezitose	No Growth	D-Tagatose	No Growth
D-Raffinose	No Growth	D-Fucose	No Growth
Starch	Growth	L-Fucose	No Growth
Glycogen	Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	No Growth	Potassium Gluconate	No Growth
D-Turanose	No Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth		

Metabolite production of *R. bovis* ASCUSDY10 was measured at 32 hours elapsed fermentation time grown on a complex media with maltose using an Agilent 1260 series HPLC with refractive index (RI) detector. The results are summarized in Table 2.2 and Appendix 002. Major fermentation products include acetate and ethanol.

Table 2.2: Metabolite Production of *R. bovis* ASCUSDY10 on Complex Media with Maltose	
Metabolite	**Production (g/L)**
Pyruvic acid	0
Succinic acid	0.11
Lactic acid	0.03
Glycerol	0
Acetic acid	1.84
Propionic acid	0
Butyric acid	0
Ethanol	1.84
1-Butanol	0

2.2.2 Identification of the Microorganism

2.2.2.1 Taxonomy

R. bovis ASCUSDY10 is the type strain of *Ruminococcus bovis*, a species of *Oscillospiraceae* family (Table 2.3) (Gaffney et al., 2021). The taxonomic classification was determined via both 16S rRNA gene sequencing/phylogeny (Part 2.2.2.2 and 2.2.2.3) and whole genome comparison (Part 2.2.2.4 and 2.2.2.5).

Table 2.3: Taxonomic Classification of *R. bovis*	
Kingdom	Bacteria
Phylum	Bacillota (formerly known as Firmicutes)
Class	Clostridia
Order	Eubacteriales (formerly known as Clostridiales)
Family	*Oscillospiraceae* (formerly known as *Ruminococcaceae*)
Genus	*Ruminococcus*
Species	*bovis*

It is important to mention that two distinct groups belonging to two different families, *Oscillospiraceae* and *Lachnospiracea*, used to share the same genus name *Ruminococcus* (Ezaki 2015; Liu et al. 2008). The *Lachnospiraceae* lineage *Ruminococcus* was recently reclassified as *Mediterraneibacter*, which includes *Mediterraneibacter* (*Ruminococcus*) *torques*, *Mediterraneibacter* (*Ruminococcus*) *gnavus*, *Mediterraneibacter* (*Ruminococcus*) *faecis*, and *Mediterraneibacter* (*Ruminococcus*) *lactaris* (Togo et al., 208; Oren et al., 2019; personal communication with Dr. Oren). The *Oscillospiraceae* lineage retained the

Rumincoccus name, and includes *Ruminococcus flovefaciens, Ruminococcus albus, Ruminocaccus bromii,* and *Ruminococcus callidus. R. bovis* belongs to the *Oscillospiraceae* lineage and is significantly different from the *Lachnospiroceae* lineage group (Ezaki 2015; Gaffney et al., 2021).

2.2.2.2 16S rRNA Gene Sequence Comparison

The 16S rRNA gene was amplified from the strain using 27F and 543R primers and paired end sequenced [2x300 base pairs (bp)] using an Illumina Miseq (Muyzer, de Waal, and Uitterlinden 1993). The resulting 16S rRNA gene fragment was quality trimmed and compared to the existing sequences in NCBI using the Basic Local Alignment Search Tool (BLAST) to establish the identity of the strain. Details of the analysis are provided in Appendix 003A. The amplified 16S rRNA gene fragment is 100% identical to *R. bovis* strain JE7A12. Other species that are closely related to *R. bovis* ASCUSDY10 include *Ruminococcoides bili* (93.38%) and *Ruminoccoccus bromii* (93.15%). The 16S rRNA analysis unambiguously identifies the microbial strain presented in this dossier as *R. bavis,* as it is the only match above the 98.2-99% similarity threshold typically used to define species (Meier-Kolthoff et al., 2013; Kim et al., 2014). Results of the alignment can be found in Table 2.4.

Table 2.4: 16S rRNA alignment to *R. bovis* ASCUSDY10 16S rRNA by BLAST

Genus species (Genbank accession #)	**Identity (%)**	**Coverage (%)**
Ruminococcus bovis type strain (JE7A12) (deposited by Native Microbials)	100	100
Ruminococcoides bili strain (IPLA60002)	93.38	99
Ruminococcus bromii strain (ATCC 27255)	93.15	94
Clostridium leptum strain (DSM 753)	91.79	99
Ruminococcus bromii Rb (MT152631)	91.2	95
Ruminococcus bromii YE282 (DQ882649)	91.1	96
Anaeromassilibacillus senegalensis strain (mt9)	90.9	99
Ruminococcus bromii L2-63 (EU266549)	90.3	95
Butyrcicoccus pullicoecorum 25-3 (NR044490)	90.26	98

2.2.2.4 Whole Genome Sequence Assembly and Annotation

Genomic DNA was isolated from a pure culture of *R. bovis* ASCUSDY10 and sequencing libraries were prepared using the (b) (6), (b) (4)). The resulting libraries were paired-end sequenced (1x300bp) on an (b) (4) and in parallel, long-read libraries were prepared from the same extracted DNA using (b) (6), (b) (4)) following the protocol outlined by (Jain et al., 2018) and 1D sequenced on the (b) (6), (b) (4)) (Jain et al. 2018). The genome was assembled through hybrid methods utilizing both short and long reads for scaffold building and errors correction. Read quality and genome coverage was evaluated

using (b) (4) data and (b) (4) data. The complete genome sequence was assembled into one chromosome with a length of 2,440,231 bp and a GC content of 34.74%. Assembly statistics can be found in Table 2.5. Additional details are provided in Appendix 003B.

Protein coding genes were predicted using GLIMMER2 (Delcher 1999) and through an iterative process of annotating putative genes using the FIGfams database (Delcher 1999; Meyer, Overbeek, and Rodriguez 2009). To identify protein coding open reading frames of potential genes, contigs were first filtered of all potential tRNA coding genes (Lowe and Eddy 1996) and rRNA genes (Aziz et al. 2008). The process yielded 2,278 coding sequences from the *R. bovis* ASCUSDY10 genome.

The assembled genome has been deposited at NCBI under accession number CP039381 (Appendix 003C).

Table 2.5: Assembly Statistics for *R. bovis* ASCUSDY10	
# of Contigs	1
# of Contigs ≥ 5,000 bp	1
Longest Contig (bp)	2,440,231
Assembly Length	2,440,231
N50	2,440,231
N75	2,440,231
GC%	34.74

2.2.2.5 Whole Genome Sequence Comparison

To determine the relatedness of *R. bovis* ASCUSDY10 to other neighboring species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *R. bovis* ASCUSDY10 were selected by full length 165 rRNA similarity as well as phylogenetic distance and downloaded from the NCBI database, including the *Oscillospiraceae* lineage *Ruminococcus* species and the *Lachnospiraceae* lineage *Mediterraneibacter* (formerly *Ruminococcus*) species. (b) (4) was used to generate the alignments for ANI on the basis that this software is adept at aligning highly similar sequences and is more stringent than most other aligners such as BLAST (Kurtz et al. 2004). Results for the (b) (4) alignment can be found in Table 2.6. Because (b) (4) uses aligner that is better suited to comparing genomes that are similar to each other. (b) (4) which employs Usearch for genome alignment, was selected to generate a second set of alignments for its improved performance on incomplete and distant genomes when compared to (b) (5), (b) (4), (b) (7)(A) (Palmer et al. 2020; Lee et al. 2016). Results for the OrthoANIu alignment can be found in Table 2.7.

ANI analysis unambiguously identifies the strain presented in this dossier as *Ruminococcus bovis,* as it is the only match by both (b) (4) or (b) (4) within the 95% identity cutoff with substantial coverage of the genome (100% ANI, 100% coverage) (Yoon et al. 2017; Goris et al. 2007; Richter and Rosselló-Móra

2009). The next best species match by both methods was to an unnamed and uncultured *Eubacterium* at 95.5% ANI and 88.7% coverage.

Table 2.6: Average Nucleotide Identity (ANI) of Related Species to *R. bovis* ASCUSDY10 by (b) (4)		
Genus species (assembly)	ANI (%)	Coverage (%)
Ruminococcus bovis JE7A12^T (GCA_005601135)	100	100
Eubacterium sp. (GCA_000437975)	95.5	88.7
Ruminococcus bromii (GCA_900101355)	87.8	2.12
Ruminococcus sp. (GCA_000433495)	85.2	1.96
Eubacterium sp. (GCA_000436775)	81.8	1.94
Ruminococcaceae bacterium P7 (GCA_900100595)	91.7	0.70
Ruminococcus flavefaciens (GCA_000518765)	86.2	0.19
Ruminococcus champanellensis (GCA_000210095)	88.6	0.16
Ruminococcus callidus (GCA_000468015)	88.2	0.13
Mediterroneibacter (*Ruminococcus*) *lactaris* (GCA_000155205)	79.8	0.11
Mediterroneibacter (*Ruminococcus*) *gauvreauii* (GCA_000425525)	99.1	0.08
Ruminococcus albus (GCA_000179635)	84.6	0.06
Mediterroneibacter (*Ruminococcus*) *torques* (GCA_000153925)	80.1	0.06
Mediterraneibacter (*Ruminacaccus*) *gnavus* (GCA_000526735)	80.2	0.05

Table 2.7: Average Nucleotide Identity (ANI) of Related Species to *R. bovis* ASCUSDY10 by (b) (4)		
Genus species (assembly)	ANI (%)	Coverage (%)
Ruminococcus bovis (JE7A12^T) (GCA_005601135.1)	100	100
Eubacterium sp. (GCA_000437975)	95.4	64.7
Eubacterium sp. (GCA_000436775)	69.9	22.0
Ruminococcus sp. (GCA_000433495)	70.5	21.0
Ruminococcus bromii (GCA_900101355)	70.9	13.9
Mediterraneibacter lactaris (GCA_000155205)	66.8	4.5
Ruminococcaceae bacterium P7 (GCA_900100595)	71.2	4.3
Mediterraneibocter torques (GCA_000153925)	66.7	3.9
Ruminococcus flovefaciens (GCA_000518765)	67.6	3.8
Ruminococcus callidus (GCA_000468015)	67.2	3.4
Ruminococcus champanellensis (GCA_000210095)	67.3	3.3
Ruminococcus albus (GCA_000179635)	67.9	3.0
Mediterraneibacter gnavus (GCA_000526735)	67.7	2.4
Ruminocaccus gauvreauii (GCA_000425525)	66.5	1.6

2.2.2.6 Summary and Conclusions

Both 16S rRNA and whole genome sequencing analysis confirm the phylogenetic placement of ASCUSDY10 as *Ruminococcus bovis*, a species affiliated with the genus *Ruminococcus* of the *Oscillaspiroceae* lineage. Further, *R. bovis* ASCUSDY10 has been comprehensively described and characterized in a manuscript that has been published by the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (Gaffney *et al*, 2021).

2.2.3 Plasmid Analysis

R. bovis ASCUSDY10 does not contain any plasmids. The assembly graph for the *R. bovis* ASCUSDY10 assembly was analyzed by Bandage (Wick *et al.* 2015) to confirm that the *R. bovis* ASCUSDY10 genome contains 1 circular chromosome with no extrachromosomal fragments (Figure 2.4). Therefore, *R. bovis* ASCUSDY10 genome is complete and does not contain any plasmids.

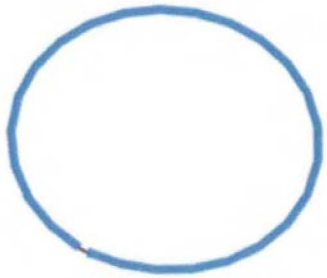

Figure 2.4 R. bovis ASCUSDY10 Assembly Graph as Generated by Bandage

2.2.4 *In-vitro* and *In-silico* Analysis of Antibiotic Susceptibility

Phenotypic antimicrobial resistance testing was conducted on *R. bovis* ASCUSDY10 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials of relevance to human and veterinary medicine. The full study report is provided in Appendix 004. The results were evaluated against the microbiological cut-off values reported by the European Food Safety Authority for "other gram-positive bacteria" (European Food Safety Authority (EFSA) 2018), as well as the resistant breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). The MIC values reported for *R. bovis* ASCUSDY10 were equal, or lower than, the cut-off values and breakpoints established by EUCAST and/or CLSI for all antimicrobials except for tetracycline. MIC values were in the intermediate range established by CLSI for tetracycline. MIC values reported for *R. bovis* ASCUSDY10 were higher than the cut-off values and breakpoints established by EFSA for tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin (Table 2.8).

It should be noted that susceptibility to aminoglycosides and macrolides decrease significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al. 2016). As such, classifications set forth by EFSA are for general gram-positive organisms and should not be applied to *R. bovis* ASCUSDY10 due to the microorganism's anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Tetracycline resistance is not uncommon among ruminal derived organisms, and recent studies have shown that tetracycline resistance is widespread amongst diverse taxa in the rumen (Sabino et al., 2019).

Table 2.8: *R. bovis* ASCUSDY10 Antimicrobial Susceptibility including EUCAST and CLSI Breakpoints					
Antibiotic	Range Tested (ug/mL)	MIC (ug/mL) of *R. bovis* ASCUSDY10	CLSI Interpretation	EUCAST Interpretation	EFSA Interpretation
Clindamycin	0.03 – 32	0.06	S	S	S
Chloramphenicol	0.5 – 64	2	S	S	S
Ampicillin	0.5 – 128	<0.5	S	S	S
Tetracycline	0.0625 – 64	8	I	N/A	R
Vancomycin	0.125 – 32	2	N/A	S	S
Gentamicin	0.5 – 32	> 32	N/A	N/A	R
Kanamycin	0.5 – 64	> 64	N/A	N/A	R
Streptomycin	0.5 – 64	> 64	N/A	N/A	R
Erythromycin	0.5 – 16	8	N/A	N/A	R

To evaluate the presence of antimicrobial resistance genes in the *R. bovis* ASCUSDY10 genome, amino acid sequences from coding regions were aligned to the PATRIC database. Included in the PATRIC database is the Comprehensive Antibiotics Resistance Database (CARD) and NCBI's National Database of Antibiotic Resistant Organisms (NDARO) for assessing antimicrobial resistance. In addition to the protein sequences from the databases, PATRIC has compiled protein hits to CARD and NDARO from 331,756 bacterial genomes and included those as redundant gene entries as a means to understand the global distribution of antimicrobial resistance proteins across diverse taxa isolated from a wide range of environments and hosts. Antimicrobial resistance was further explored using the ResFinder web server (Zankari et al. 2012) and BLASTp alignment to the NCBI AMR database as used by AMRFinder (Note: this database differs from NARDO used by PATRIC) (Feldgarden et al. 2019). Between these databases there are a total of 30,748 protein sequences and 70 sequences from *Ruminococcus*. Characteristics of each database can be found in Table 2.9.

Table 2.9: Characteristics of Databases Used to Assess Antimicrobial Resistance				
Database Name	Total Entries	*Ruminococcus* Entries	*R.bovis* Entries	Contains Redundant Entries
CARD (PATRIC)	17,559 (2,227 non redundant proteins	37 (8 non redundant proteins)	No	Yes
NDARO (PATRIC)	5,138 (4,004 non redundant proteins)	30 (9 non redundant proteins)	No	Yes
ResFinder	3,105	0	No	No
AMRFinder Plus	6,946	3	No	No

While there are no widely accepted cutoffs for detecting antimicrobial resistance at the whole genome level, many studies examining antimicrobial gene protein homologies at the whole genome level utilize 80% identity and 80% coverage (Mao *et al.* 2015; Bertelli *et al.* 2017; Hu *et al.* 2013). However, to better interrogate the genetic information and minimize the false negatives, we employed a less stringent cutoff, 30% protein sequence identity and 70% coverage and/or E-value of 1E-04 (Pearson, 2013). It is also important to mention that a homology search conducted with this less stringent cutoff will lead to misidentification of proteins that are in fact not related to anti-microbial resistance. Hits were further investigated using BLASTp against the NCBI non-redundant amino acid database (nr).

Analysis of the *R. bovis* ASCUSDY10 genome identified four possible resistance genes at high protein similarity and coverage (see Tables 2.10 to 2.12). These matches are further explained below:

tet(W): The antimicrobial gene in question is a 100% match to the tetracycline resistance gene, tet(W), in both the ResFinder and NCBI AMR databases and a 99% match to the same gene in the NDARO database. Tet(W) confers resistance to tetracycline through ribosomal protection (Aminov, Garrigues-Jeanjean, and Mackie 2001). The tet(W) gene is a ubiquitous gene in the bacterial population of the gastrointestinal microbiome of ruminants, humans, and other farm animals (Pal et al. 2016; Joyce et al. 2019; Sabino et al. 2019). Beside tet(W), no other antimicrobial resistance genes were identified.

Tet(36): The Tetracycline resistance ribosomal protection protein Tet(36) (Whittle et al., 2003) belongs to a class of ribosome protection proteins against tetracycline. Although, its amino acid identity is low (47.14%), together with tet(W) reported above, it may contribute to the observed tetracycline resistance.

CatB: The CatB protein is a type B chloramphenicol O-acetyltransferase which can confer resistance against chloramphenicol (Huang et al., 2017). The low similarity (36%) and low coverage (30%) is consistent with the observed chloramphenicol susceptibility *in vitro* (Table 2.8). Therefore, this protein does not contribute to antibiotic resistance in *R. bovis* ASCUSDY10.

NimB: The NimB proteins are believed to contribute to resistance to nitroimidazole antibiotics (Leiros *et al.*, 2004). A further analysis against the NCBI nr database identified the protein sequence as part of the pyridoxamine 5'-phosphate oxidase superfamily, which participates in the initial formation of pyridoxine (vitamin B6) and pyridoxal phosphate. Even though pyridoxamine 5'-phosphate oxidase superfamily shares <50% similarity with Nitroimidazole reductases, it has never been reported to carry out the same functions. Thus, this protein is unlikely to contribute to antibiotic resistance in *R. bovis* ASCUSDY10.

Table 2.10: *R. bovis* ASCUSDY10 Antimicrobial Resistance by PATRIC								
Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	Identity	E-Value
NDARO	*Bifidobacterium longum*	Tet(W)	Tetracycline resistance, ribosomal protection	MULTISPECIES: tetracycline resistance ribosomal protection protein Tet(W)	100	100	99	0.0

Table 2.11: *R. bovis* ASCUSDY10 Antimicrobial Resistance by ResFinder				
Gene	Identity	Query Coverage	Function	Accession number
tet(W)	100	100% (1920/1920)	Tetracycline Resistance	AJ427422

Table 2.12: *R. bovis* ASCUSDY10 Antimicrobial Resistance by NCBI AMR BLASTp				
Gene	e-value	Percent Identity	Query Coverage	Subject Coverage
tet(W)	0	100	100	100
Tet(36) Tetracycline resistance ribosomal protection protein	1e-027	47.14	98	98
CatB	1e-11	36.25	30	30
NimB	1e-41	46.01	100	100

2.2.4.4 Section Summary

In vitro MIC testing demonstrated that *R. bovis* ASCUSDY10 is resistance to gentamicin, kanamycin, streptomycin, tetracycline, and erythromycin. Resistance to aminoglycosides and macrolides like gentamicin, kanamycin, streptomycin, and erythromycin is reflective of *R. bovis* ASCUSDY10 being an anaerobe rather than any organism-specific resistance mechanism or genotype. Consistent with the *in vitro* testing, genomic analysis indicates the presence of tet(W) in *R. bovis* ASCUSDY10, a gene implicated in tetracycline resistance. This is not uncommon, since many members of the rumen microbial community have tetracycline resistance genes. *R. bovis* ASCUSDY10 is susceptible to clindamycin, chloramphenicol, ampicillin, and vancomycin, suggesting that should *R. bovis* ASCUSDY10 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics.

2.2.5 Antimicrobial Production

R. bovis ASCUSDY10 supernatant obtained post fermentation was tested for inhibitory activity against reference strains known to be susceptible to a range of antibiotics. No zones of inhibition were observed indicating that the strain is not an antimicrobial producer. Further details of the study are provided in Appendix 005.

2.2.6 Pathogenicity and Virulence

To assess the presence of virulent and pathogenic genes, amino acid sequences from coding regions identified in Part 2.2.2.4 were queried against several databases. All applicable, publicly available databases were used to identify potential pathogenic genes. The characteristics of these databases are described in Table 2.13 and below.

- The PATRIC (BV-BRC) database has compiled relevant genes from external databases including Victors, Virulence Factors Database (VFDB), and the PATRIC_VF database. The total number of protein sequences in the PATRIC is 127,616 bacterial protein coding genes belonging to 810,928 genomes, with 3,027 genome entries from *Ruminococcus* and one from *R. bovis*. Redundant gene entries (e.g. the same virulence factor showing up in multiple microbial species) are included to understand the global distribution of pathogenicity and virulence associated proteins across diverse taxa isolated from a wide range of environments and hosts.
- Both the VFDB and Victors databases were downloaded and queried independently of PATRIC to ensure features in these databases that were excluded by PATRIC were represented. Victors and VFDB contain 27,370 sequences, none of which originate from *Ruminococcus.*
- BLASTp alignment to the Pathogen-Host Interaction Database (Phi-BASE) (Urban et al. 2015) were also utilized to assess the potential pathogenicity and virulence of *R. bovis* ASCUSDY10. The Phi-BASE database has 6,010 sequences with no entries from *Ruminococcus*.
- IslandViewer4 web server was used to identify any potential genomic islands that may contain virulence factors (Bertelli et al. 2017). The database contains 21,106 non-redundant pathogenicity islands including 7 from *Ruminococcus*.
- PathogenFinder conducts database independent analysis, which uses a model trained with protein sequences from 886 whole genome sequences including one *Ruminococcus* genome, to predict pathogenicity (Cosentino et al. 2013). The PathogenFinder model predicts pathogenicity based on matches to proteins found differentially in pathogenic and non-pathogenic bacteria regardless of their annotated function. Therefore, a single hit to a protein found in pathogenic species does not necessarily suggest the query organism is virulent or pathogenic, but a collection of hits to proteins uniquely found in pathogens could be enough for PathogenFinder to deem the organism pathogenic, even if the proteins are not traditionally implicated in virulence or pathogenicity. The program allows the organism to be evaluated more holistically and enables the evaluation of proteins that are potentially involved in virulence and pathogenicity beyond well annotated virulence factors such as toxins.

Table 2.13: Characteristics of Databases Used to Assess Virulence and Pathogenicity				
Database Name	Number of Entries	Number of *Ruminococcus* Entries	*R. bovis* Entries	Contains Redundant Protein ID entries
BV-BRC (PATRIC)	810,928 (Genomes)	3,027 (Genomes)	Yes	No
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	13 (6 non-redundant proteins)	No	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	8 (3 non redundant proteins)	No	Yes
PATRIC_VF	38,791(1,570 non-redundant proteins)	0	No	Yes
Victors	4,965 (non-redundant proteins)	0	No	No
VFDB	22,405	0	No	Yes
Phi-Base	6,010	0	No	No
IslandViewer4	21,106 (pathogenicity islands)	7 (pathogenicity islands)	No	No
PathogenFinder	N/A	N/A	N/A	N/A

Published studies often employ protein alignments to explore the presence of pathogenicity/virulence factors in microbial genomes (Liang *et al.* 2020; Hu *et al.* 2013; Abril *et al.* 2020; Deng *et al.* 2021; Rojas-Estevez *et al.* 2020; Pan *et al.* 2020). In our investigation of potential pathogenicity/virulence factors in DY10, a 70% protein similarity and 70% query coverage interrogation was applied and no virulence proteins of concern were discovered. To be thorough, however, a lower threshold (30% for protein similarity, 70% query coverage, and/or an E-value threshold of 1E-04) was applied to re-assess protein homologies and evaluate the safety of DY10 (Pearson, 2013). Lower protein alignment thresholds will result in numerous false positives (misidentify non-pathogenic/non-virulent protein as pathogenic/virulent). To address this, we conducted additional BLASTp analyses against the NCBI nr database to verify the identity of the protein hits. If the BLASTp top hits against the NCBI nr database yielded different protein annotations compared to those from the databases in Table 2.13, using the same homology thresholds, the protein hits were considered misidentified and unlikely to be pathogenicity/virulence factors. The results are shown in Table 2.14-2.16.

No genes involved in toxin synthesis, pathogenicity, or virulence were identified by PATRIC or IslandViewer. One protein from a pathogen was identified in *R. bovis* ASCUSDY10 genome by PathogenFinder, a TrsE-like protein. TrsE (also known as traE) is a membrane bound ATPase that is thought to be involved in type IV secretion systems (T4SSs) (Bai, Fazzolari, and Hogenhout 2004; Casu et al. 2016) (Table 2.16). T4SSs act to transport proteins, DNA via conjugation, and other macromolecules across cell

membranes in both gram positive and gram negative bacteria (Bai, Fazzolari, and Hogenhout 2004; Ilangovan, Connery, and Waksman 2015; Casu et al. 2016; Goessweiner-Mohr et al. 2013; Wallden, Rivera-Calzada, and Waksman 2010). Despite the identification of this protein, no other proteins associated with T4SSs were encoded by the *R. bovis* ASCUSDY10 genome. A BLAST search of the TrsE-like protein in question revealed homologues in pathogenic and non-pathogenic species, as well as in other *Ruminococcus* (Table 2.17) indicating that the protein alone does not impart pathogenicity. Ultimately, PathogenFinder deemed *R. bovis* ASCUSDY10 to "not be predicted as a human pathogen". The results for these analyses can be found in Tables 2.14 -2.17.

Table 2.14: Significant matches Between the Victors Virulence Database and *R. bovis* ASCUSDY10	
Database	*R. bovis* ASCUSDY10
Protein Hits to Victors (PATRIC)	0
Protein Hits to Victors	0
Protein Hits to VFDB (PATRIC)	0
Protein Hits to VFDB	0
Protein Hits to PATRIC_VF	0
Protein Hits to Phi-Base	0
Pathogenicity Island Hits in IslandViewer	0
Hits to Proteins from Pathogens in PathogenFinder	1

Table 2.15: PathogenFinder evaluation for *R. bovis* ASCUSDY10	
Gene Matches	2
Proteins from Pathogens Matched	1
Proteins from Non-Pathogens Matched	1
Predicted as Human Pathogen?	No

Table 2.16: *R. bovis* ASCUSDY10 Hits to Pathogenic Genes in PathogenFinder	
Gene	TrsE-like protein
Genbank Accession Number	ADE30946
Source Organism	*Streptococcus suis* GZ1
Percent Identity	84.7

Table 2.17: BLASTp Hits to TrsE-like protein from *Streptococcus suis* GZ1					
Organism	Protein Name	Genbank Accession Number	Percent Identity	Query Coverage	Known Pathogen?
Caproiciproducens galactitolivorans	ATP-binding protein	WP_135660690	99.9	100	No
Enterocloster clostridioformis	ATP-binding protein	WP_003525203	99.6	100	No
Clostridium indicum	ATP-binding protein	WP_117416073	99.4	100	No
Clostridium leptum	ATP-binding protein	WP_117818845	99.5	99	No
Mediterraneibacter (Ruminococcus) gnavus	Type IV secretory pathway VirB4	CUN32737	97.2	99	No
Ruminococcus bromii	ATP-binding protein	WP_101069761	93.7	100	No
Lactobacillus murinus	ATP-binding protein	WP_089135567	93.7	100	No
Clostridioides difficile	TraE protein	VIH56643	91.7	99	Yes

2.2.7 Toxigenicity

In addition to pathogenicity/virulence factors, microbial toxins also contribute to safety concerns. Because toxigenic proteins often feature multiple domains, with only one of these domains responsible for the toxin's harmful effects, two toxin databases were used to identify potential toxigenic proteins in *R. bovis* ASCUSDY10, using the same 30% protein sequence identity with 70% query coverage and/or E-value cutoff of 1E-04 that was used previously (Pearson, 2013; Negi *et al.* 2017; Xie and Fair 2021). The two databases used to assess toxigenicity are shown in Table 2.18. One of the databases was the VFDB core data database, which consists of 290 exotoxin and exoenzyme sequences. The other database was the Database for Bacterial ExoToxins (DBETH), which contains the sequence, structure, interaction network and analytical results for 229 toxins (Chakraborty et al., 2012). However, as shown in table 2.18, none of the databases had queries specific to *Ruminococcus* species.

Table 2.18: Characteristics of Databases Used to Assess Toxigenicity				
Database Name	Number of Entries	Number of *Ruminococcus* Entries	*R. bovis* Entries	Contains Redundant Protein ID entries
VFDB	290 (toxin sequences)	0	No	Yes
DBETH	229 (toxin sequences)	0	No	No

Using the above-mentioned methods and databases, 24 unique matches (25 total with 1 duplicated match) were identified in *R. bovis* ASCUSDY10. Full results can be found in Tables 2.19 and 2.20. Each of the putative toxin proteins identified were queried against the NCBI (nr) database using BLASTp search tool to confirm annotations and assess the distribution of the protein globally. The results from the BLASTp search can be found in Table 2.21.

Table 2.19: *R. bovis* ASCUSDY10 Significant Protein Alignments to VFDB Toxin Sequences							
ASCUSDY10 protein ID	**VFDB ID**	**VFDB Annotation**	**Source Organism**	**Subject Coverage (%)**	**Query Coverage (%)**	**Identity (%)**	**E-Value**
peg.529	VFG005775	ABC (ATP-binding cassette) Transporter, CylA	*Streptococcus agalactiae* NEM316	100	99	39.2	1.00E-57
peg.1416	VFG050215	cell wall-binding cysteine protease, Cwp84	*Clostridium difficile* 630	59	81	32.3	3.00E-55
peg.204	VFG005787	putative 3-ketoacyl-ACP synthase, CylI	*Streptococcus agalactiae* NEM316	56	98	30.9	2.00E-45
peg.530	VFG005778	ABC (ATP-binding cassette) transporter, CylB	*Streptococcus agalactiae* NEM316	90	88	34.2	2.00E-42
peg.891	VFG040705	Hemolysin C,TlyC	*Rickettsia typhi* str. Wilmington	67	70	32.4	3.00E-28
peg.731	VFG000841	Hemolysin B, hlyB	*Escherichia coli* O157:H7 str. EDL93	32	63	37.2	3.00E-27
peg.2055	VFG038918	RTX toxin transporter, ATPase protein	*Aeromonas hydrophila* subsp. hydrophila ATCC 7966	29	91	33.8	2.00E-22
peg.216	VFG005775	precolibactin export MATE transporter ClbM	*Klebsiella pneumoniae* subsp. pneumoniae 1084	72	74	24.6	2.00E-22
peg.1184	VFG050117	4'-phosphopantetheinyl transferase, CesP	*Bacillus cereus* AH187	90	96	28.3	9.00E-16
peg.969	VFG050115	Cereulide synthetase A, CesA	*Bacillus cereus* AH187	3	77	38.0	5.00E-15
peg.1430	VFG049158	colibactin biosynthesis amidase ClbL	*Klebsiella pneumoniae* subsp. pneumoniae 1084	47	46	30.3	2.00E-14
peg.2072	VFG050113	ABC transporter ATP-binding protein, CesC	*Bacillus cereus* AH187	29	55	41.2	1.00E-12
peg.402	VFG037203	phosphatidylserine/ Phospholipase D /cardiolipin synthase	*Acinetobacter baumannii* ACICU	76	61	23.1	1.00E-12
peg.312	VFG045470	cytolysin regulator R2	*Enterococcus faecalis* str. MMH594	65	9	46.9	2.00E-12
peg.541	VFG002177	cytolysin activator (clyA)	*Enterococcus faecalis* str. MMH594	55	18	25.8	8.00E-06
peg.1128	VFG002177	esterase of alpha/beta hydrolase family	*Rickettsia typhi* str. Wilmington	5	10	62.1	1.00E-05
peg.709	VFG045334	LlsX	*Listeria Innocua* SLCC6294	81	88	30.6	8.00E-05
peg.2056	VFG002196	polysaccharide lyase	*Enterococcus faecalis* V583	4	7	42.4	9.00E-05

Table 2.20: *R. bovis* ASCUSDY10 Significant Protein Alignments to DBETH Toxin Sequences							
ASCUSDY10 protein ID	**DBETH ID**	**DBETH Annotation**	**Source Organism**	**Subject Coverage**	**Query Coverage**	**% Identity**	**E-Value**
peg.535	Q897Y4	Hemolysin III	*Clostridium tetani*	96	94	51.2	2.00E-66
peg.1629	D0HWK0	Hemolysin	*Vibrio cholerae*	84	59	28.0	6.00E-36
peg.1931	Q73VP2	LepB	*Mycobacterium paratuberculosis*	31	35	33.3	1.00E-05
peg.402	Q9ZCD8	Phospholipase D	*Rickettsia prowazekii*	46	18	29.8	2.00E-05
peg.1732	Q897D0	Zn-dependent peptidase, insulinase family	*Clostridium tetani*	34	69	21.8	2.00E-05
peg.1356	Q9EZE7	Serine protease espC	*Escherichia coli*	7	38	29.7	4.00E-05
peg.2251	B0HB09	Protein kinase YopO	*Yersinia pestis*	21	19	23.2	8.00E-05

Table 2.21: BLASTp Matches in NCBI to Potential Toxin Sequences in the *R. bovis* ASCUSDY10 Genome				
ASCUSDY10 protein ID	**Organisms providing best match by BLAST**	**Annotation of closest related protein in NCBI**	**Identity (%)**	**Query Coverage (%)**
peg.529	*Paenibacillus crassostreae*	ABC transporter ATP-binding protein	64.8	100
peg.1416	*Ruminococcus bromii*	lectin like domain-containing protein	36.6	97
peg.204	*Ruminococcus bromii*	beta-ketoacyl-ACP synthase II	75.4	99
peg.530	*Sporobacter termitidis*	ABC transporter permease	48.8	99
peg.891	*Caproiciproducens galactitolivorons*	hemolysin family protein, HlyC/CorC family transporter	55.5	92
peg.731	*Ruminococcus bromii*	Predicted Zn-dependent peptidase	50.8	99
peg.2055	*Roseburia hominis*	ABC transporter ATP-binding protein	68.8	96
peg.216	*Ruminococcus bromii*	MATE family efflux transporter	66.4	100
peg.1184	*Clostridium porci*	4'-phosphopantetheinyl transferase superfamily protein	33.5	89
peg.969	*Acetivibrio straminisolvens*	AMP-binding protein	43.5	97
peg.1430	*Ruminococcus bromii*	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A	78.4	100
peg.2072	*Ruminococcus bromii*	ABC transporter ATP-binding protein	88.9	100

Table 2.21 (cont.): BLASTp Matches in NCBI to Potential Toxin Sequences in the *R. bovis* ASCUSDY10 Genome				
ASCUSDY10 protein ID	**Organisms providing best match by BLAST**	**Annotation of closest related protein in NCBI**	**Identity (%)**	**Query Coverage (%)**
peg.402	*Allobaculum stercoricanis*	cardiolipin synthase	57.0	100
peg.312	*Eubacterium ruminantium*	putative transcriptional regulator	85.7	98
peg.541	*Mediterraneibacter (Ruminococcus) gnavus*	S8 family serine peptidase	31.7	99
peg.1128	*Blautia glucerasea*	patatin family protein	43.4	99
peg.709	*Clostridium innocuum*	DUF5963 family protein	55.9	100
peg.2056	*Longicatena caecimuris*	Ig-like domain-containing protein	32.7	58
peg.535	*Ruminococcus bromii*	hemolysin III family protein	73.5	100
peg.1629	*Ruminococcus bromii*	hemolysin family protein	54.4	97
peg.1931	*Sharpea porci*	signal peptidase I	54.3	68
peg.1732	*Ruminococcus bromii*	pitrilysin family protein	62.9	100
peg.1356	*Ruminococcus bromii*	UDP-N-acetylglucosamine pyrophosphorylase	78.6	99
peg.2251	*Ruminococcus bromii*	serine/threonine protein kinase	57.6	94

Most of the features identified in the toxin search yielded alignments to DBETH or VFDB had better non-toxigenic matches when queried against NCBI GenBank nr database using BLASTp tool and originated from species not known to be pathogenic or pathobiontic.

- Eleven of the proteins matched to features from non-pathogenic *Ruminococcus bromii.*
- Another 11 of the features were re-identified as transporters and regulatory proteins in organisms that are a part of the normal flora in a diverse group of animals including humans.
- Two of the features most closely match proteins from *Mediterraneibacter gnavus* and *Clostridium innocuum* which are human commensal organisms and rare pathobionts, the former being associated with inflammation events in humans. These two features are investigated more thoroughly below.
- Four features of *R. bovis* ASCUSDY10 matched to proteins similar to hemolysin in non-pathogenic bacteria (VFDB peg.731, 1629, and 535 matched to the proteins in *R. bromii*; peg.891 matched to a protein in *Caproiciproducens galactitolivorans*). Hemolysin analysis is investigated more thoroughly below.

To further investigate potential risks, a detailed evaluation of these 6 features was performed:

LlsX: The feature from *R. bovis* ASCUSDY10 (peg.709), which shares a similarity to a feature from *Clostridium inocuum* (55.9% identity, 100% coverage), is annotated as LlsX from *Listeria innocua* in VFDB (31% identity, 88% coverage). This annotation differs from the annotation of the protein match from

Clostridium inocuum, which is annotated broadly as a DUF5963 family protein with unknown functions. This suggests that the annotation of peg.709 as LlsX is likely incorrect.

Furthermore, in *Listeria,* LlsX encodes for a protein which enhances the expression of the bacteriocin, listeriolysin S, which is not present in *R. bovis* ASCUSDY10. Therefore, even if this feature is similar to LlsX, it is not directly responsible for pathogenicity (Maćkiw et al., 2021).

clyA: The feature from *R. bovis* ASCUSDY10 (peg.541), which shares a low similarity to a feature from *M. gnavus* (32% identity, 99% coverage), is annotated as cytolysin activator (clyA) from *Enterococcus faecalis* in VFDB (26% identity, 18% coverage). This annotation differs from the annotation of the protein match from *M. gnavus,* which is annotated broadly as a S8 family serine peptidase. This suggests that the annotation of peg.541 as clyA is likely incorrect.

Further, in infectious *Enterococcus faecalis,* cytolysin is a component of a large pathogenicity island that contributes to pathogenicity through cytotoxic activity targeting erythrocytes and macrophages (Shankar et al., 2002; Oruc et al., 2021). The clyA gene is an activator of cytolysin and not the cytolysin itself, so alone it does not directly contribute to pathogenicity.

hylB: The feature Hemolysin B/hlyB, peg.731, shares a similarity (37% identity, 63% coverage) to a protein found in *Escherichia coli* O157:H7 str. EDL93 according to VFDB (Table 2.18). Querying the protein sequence of this feature against NCBI nr database showed this protein shares a similarity (51% identity, 99% coverage) to a predicted Zn-dependent peptidase in *R. bromii* (Table 2.20). The inconsistency in annotation and the low protein sequence similarity to pathogenic *E. coli* suggests that the annotation of this feature is likely inaccurate.

TlyC: The feature Hemolysin C, TlyC, peg.891, shares a similarity (32% identity, 70% coverage) to a protein found in *Rickettsia typhi* str. Wilmington according to VFDB. However, querying this feature against NCBI nr database revealed that it is similar to hemolysin family protein or a HlyC/CorC transporter found in *C. galactitolivorans* (56% identity, 92% coverage). HlyC/CoC transporter modulates the transport of ion substrates and is not related to toxigenicity (Sun et al., 2021). Furthermore, *C. galactitolivorans* is not a known pathogen and there is no literature to support hemolytic activity in the species. The annotation of this feature is likely inaccurate.

Non-specific hemolysin: Feature, peg.1629, shares a similarity (28% identity, 59% coverage) to a hemolysin family protein found in *Vibrio cholerae* based on DBETH. Querying the protein sequence of this feature against NCBI nr database identified that this protein is more similar (54% identity, 97% coverage) to a hemolysin family protein found in *R. bromii* (Table 2.20) than those of *V. cholerae*. Although *R. bromii* is not a known pathogen and no hemolytic activity has been reported in literature, it does not guarantee the gene is inactive. Therefore, we conducted a series of *in vitro* hemolytic assays to confirm the lack of hemolytic activity in *R. bovis* ASCUSDY10 (see below).

Hemolysin III: The feature Hemolysin III from *R. bovis* ASCUSDY10 (peg.535) is annotated as Hemolysin III from *Clostridium tetani* according to DBETH (51% identity, 94% coverage). The protein sequence alignment using BLASTp reveals that peg.535 is more similar to a Hemolysin III protein found in *R. bromii*, which is a bacterium with no reported pathogenicity, virulence, and hemolytic activity (73.5% identity,

100% coverage). Hemolysin III, also referred to as yqfA, is a hemolysin containing a yqfA transmembrane domain originally identified in *Bacillus cereus* (Baida and Kuzmin 1996; Ramarao and Sanchis 2013). A search of proteins containing the yqfa domain architecture in NCBI revealed that the domain is found in proteins with functionally diverse, non-pathogenic membrane associated features (Mahu *et al* 2016). Therefore, we conducted a series of *in vitro* hemolytic assays to confirm the lack of hemolytic activity in *R. bovis* ASCUSDY10.

2.2.7.1 Hemolysis Evaluation In Vitro

A series of hemolysis assays, using various animal bloods, was conducted to confirm that *R. bovis* ASCUSDY10 exhibits no hemolytic activity under physiologically relevant conditions (see Appendix 021). The quantitative hemolysin assay consisted of three different animal bloods (ox, rabbit, and sheep) in microtiter plates using the method adapted from Riddler *et al*, 2021. All tests were incubated anaerobically at various temperatures and pH's to mimic a range of rumen-relevant normal and stress conditions. *Staphylococcus aureus* ATCC 25923 was used as positive control (beta-hemolytic) and *Staphylococcus hominis* (ATCC 27844) was used as negative control (non-hemolytic). The results of the assays are shown below in Figure 2.5. In all blood types and with all pH conditions, the assays show that no hemolytic activity was detected in *R. bovis* ASCUSDY10. This suggests that while hemolysin family proteins and hemolysin III homologs were identified in the *R. bovis* ASCUSDY10 genome at the 30% protein sequence identity with 70% query coverage level, *R. bovis* ASCUSDY10 does not exhibit a hemolytic phenotype under physiologically relevant conditions. This also confirms that the initial genome interrogation of 70% protein sequence identity with 70% query coverage, which showed no hemolysis genes of concern, was an accurate assessment.

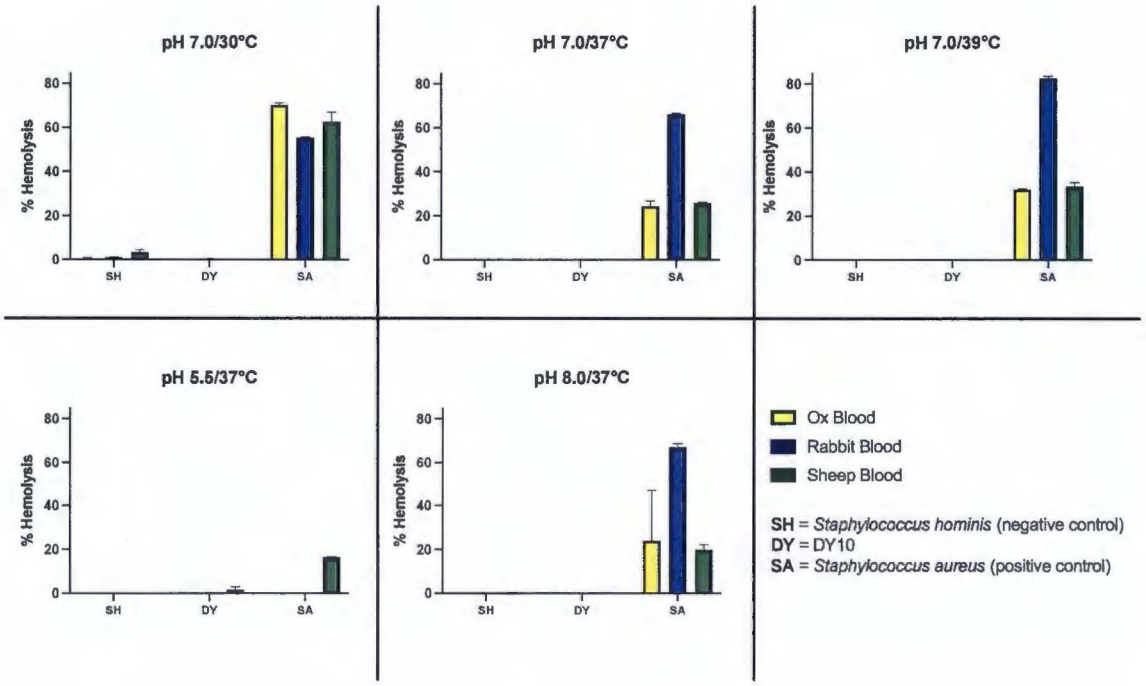

Figure 2.5 Hemolysis Assay results as calculated percent hemolysis, separated by condition and grouped by organism, with R. bovis ASCUSDY10 indicated by DY

2.2.7.2 Alignment to Genomic Islands from Mediterraneibacter gnavus

Recently, a biosynthetic cluster in *M. gnavus* (formerly group 2 *Ruminococcus*) that encodes for a potentially pro-inflammatory polysaccharide, glucorhamnan, was discovered (Henke *et al.*, 2019). Glucorhamnan is hypothesized to modulate inflammatory pathways similarly to yeast derived mannan. Although *R. bovis* ASCUSDY10 is phylogenetically distant from *M. gnavus,* it was deemed prudent to interrogate the *R. bovis* ASCUSDY10 genome for presence of the glucorhamnan gene cluster.

Protein alignment of the 23 gene biosynthetic cluster using 30% protein identity with 70% query coverage and/or an E-value of 1E-04 returned 12 protein matches to the predicted proteome of *R. bovis* ASCUSDY10. Of the 12 matches, most were low identity at a range of coverage (25%-99%). None of the alignments yielded a match greater than 65.3% identity. Additionally, the 12 features that were identified in *R. bovis* ASCUSDY10 are not co-located in a cluster as they are in *M. gnavus*. The alignment results can be found in Table 2.22.

Eleven proteins in the cluster provided no alignment to *R. bovis* ASCUSDY10. Missing features included all the putative regulatory, oligosaccharide polymerization, and polyglycerolphosphate synthesis features in the cluster. Other missing features include a putative transport, a glycosyltransferase, two cell wall remodeling proteins, a glucose priming protein, and a protein with unknown function. These missing proteins serve vital roles in the production of glucorhamnan as outlined by Henke et al.

Taken together, the relatively low identity alignments, lack of gene co-localization, and missing proteins, suggest that *R. bovis* ASCUSDY10 does not possess a viable glucorhamnan biosynthetic gene cluster.

Table 2.22: Protein Alignments between the Glucorhamnan gene cluster and *R. bovis* ASCUSDY10

ASCUSDY10 protein ID	Feature Accession #	Feature Locus Tag	Gene Product	Function	Identity (%)	Query Coverage (%)
No Match	EDN75889.1	RUMGNA_03512	hypothetical protein (carbohydrate binding domain)	Regulatory	-	-
peg.1000	EDN75890.1	RUMGNA_03513	cell envelope-like function transcriptional attenuator	Cell wall remodeling	35.0	36
No Match	EDN75891.1	RUMGNA_03514	glycosyltransferase, group 2 family protein	glycosyltransferase	-	-
No Match	EDN75892.1	RUMGNA_03515	hypothetical protein (LytR/CpsA/Psr like)	oligosaccharide polymerization	-	-
No Match	EDN75893.1	RUMGNA_03516	cell envelope-like function transcriptional attenuator	Cell wall remodeling	-	-
peg.347	EDN75894.1	RUMGNA_03517	undecaprenyl-phosphate glucose phosphotransferase	Glucose priming	39.2	83
peg.1193	EDN75895.1	RUMGNA_03518	glycosyltransferase, group 2 family protein	glycosyltransferase	35.7	35
peg.342	EDN75896.1	RUMGNA_03519	glycosyltransferase, group 1 family protein	glycosyltransferase	27.4	25
No Match	EDN75897.1	RUMGNA_03520	hypothetical protein (sporulation)	Regulatory	-	-
peg.1189	EDN75898.1	RUMGNA_03521	dTDP-4-dehydrorhamnose reductase	rhamnose biosynthesis	61.3	99
peg.1211	EDN75899.1	RUMGNA_03522	hypothetical protein (flippase like)	transport	29.8	87
No Match	EDN75900.1	RUMGNA_03523	hypothetical protein (LtaA like)	polyglycerolphosphate synthesis	-	-
peg.340	EDN75901.1	RUMGNA_03524	glycosyltransferase, group 2 family protein	glycosyltransferase	46.1	34
No Match	EDN75902.1	RUMGNA_03525	hypothetical protein	unknown	-	-
peg.467	EDN75903.1	RUMGNA_03526	ABC transporter, ATP-binding protein	transport	46.0	45.7
No Match	EDN75904.1	RUMGNA_03527	ABC-2 type transporter	transport	-	-

Table 2.22 (cont) : Protein Alignments between the Glucorhamnan gene cluster and *R. bovis* ASCUSDY10						
ASCUSDY10 protein ID	**Feature Accession #**	**Feature Locus Tag**	**Gene Product**	**Function**	**Identity (%)**	**Query Coverage (%)**
peg.1190	EDN75905.1	RUMGNA_03528	dTDP-4-dehydrorhamnose 3,5-epimerase	rhamnose biosynthesis	65.3	99
peg.1394	EDN75906.1	RUMGNA_03529	glucose-1-phosphate thymidylyltransferase	rhamnose biosynthesis	28.4	62
peg.1188	EDN75907.1	RUMGNA_03530	dTDP-glucose 4,6-dehydratase	rhamnose biosynthesis	53.9	99
No Match	EDN75908.1	RUMGNA_03531	N-acetylmuramoyl-L-alanine amidase	Cell wall remodeling	-	-
peg.900	EDN75909.1	RUMGNA_03532	glycosyltransferase, group 2 family protein	glycosyltransferase	50.3	96
No Match	EDN75910.1	RUMGNA_03533	arylsulfatase	polyglycerolphosphate synthesis	-	-
No Match	EDN75911.1	RUMGNA_03534	hypothetical protein (UTP-glucose-1-phosphate uridylyltransferase)	Glucose priming	-	-

2.2.7.3 Section Summary

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *R. bovis* ASCUSDY10 (Table 2.13). Comprehensive alignment of the *R. bovis* ASCUSDY10 predicted proteome to these databases yielded 25 unique hits at identity cutoffs of 30% with at least 70% query coverage and/or E-value cutoff of 1E-04.

Among the 25 unique matches, a TrsE-like protein, similar to those found in the pathogenic species *Streptococcus suis*, was identified in the *R. bovis* ASCUSDY10 genome by PathogenFinder. Literature shows that the functionality of TrsE protein is associated with type IV secretion system, which is not present in the *R. bovis* ASCUSDY10 genome (Bai, Fazzolari, and Hogenhout 2004; Casu *et al.* 2016). Additionally, a BLAST search revealed that homologous to the TrsE-like feature are found in other non-pathogenic species (including *Ruminococcus*). Ultimately, *R. bovis* ASCUSDY10 was deemed non-pathogenic by PathogenFinder.

The other 24 unique matches were questionable alignments to protein toxins and were evaluated in further detail. Eighteen of the proteins in question more closely matched proteins with different annotated function, from species not known to be pathogenic or pathobiontic, or both. Six features, cytolysin activator (clyA), llsx, and 4 hemolysin like proteins raised the need for further evaluation. Two of the features share low identity to clyA and llsX and are likely misannotated literature, which also shows that clyA and llsX do not contribute to pathogenicity on their own. The four hemolysin-like proteins share

similarity to pathogen hemolysins (28-51% protein identity, 59-94% coverage) but exhibit no phenotypic hemolysin activity, as seen in the *in vitro* hemolysis assays. The *in vitro* hemolysis assay confirmed that *R. bovis* ASCUSDY10 does not demonstrate hemolytic activity, suggesting that these hemolysin-like proteins are either misannotated or lack the regulatory genes to confer the hemolytic phenotype.

A recently discovered biosynthetic gene cluster from *M. gnavus* was investigated and aligned to the predicted proteome of *R. bovis* ASCUSDY10. Of the 23 genes in the cluster, 12 dispersed matches were found in *R. bovis* ASCUSDY10. The low identity alignments, lack of gene co-localization, and missing proteins, suggest that *R. bovis* ASCUSDY10 does not possess the ability to synthesize the product in question.

Taken together, no genes directly involved in pathogenesis/virulence, toxin production, or pro-inflammatory polysaccharide were identified in *R. bovis* ASCUSDY10.

2.2.7 Summary of Organism Safety Based on Genomics

Based on the above mentioned *in silico* and *in vitro* analysis results, *R. bovis* ASCUSDY10 is a non-pathogenic, non-virulent, and non-toxigenic rumen microorganism. Therefore, *R. bovis* ASCUSDY10 mediated opportunistic infections in a human or animal are highly unlikely. If *R. bovis* ASCUSDY10 mediated opportunistic infections a human or animal were to occur, the infections could be easily treated using standard antibiotics (e.g., clindamycin, chloramphenicol, ampicillin, or vancomycin). Thus, *R. bovis* ASCUSDY10 is safe for use as a direct fed microbial.

2.3 Method of Manufacture

2.3.1 Raw Materials and Processing Aids

The raw materials and processing aids used in the manufacture of fat encapsulated *Ruminococcus bovis* ASCUSDY10 are listed in Appendix 009. All raw materials used in the manufacture of *R. bovis* ASCUSDY10 have a history of use in the industrial food and feed fermentation processes, and are considered by Native Microbials to be safe and suitable for use in the manufacture of feed ingredients in the U.S.

2.3.2 Manufacturing Process

A schematic overview of the manufacturing process of *R. bovis* ASCUSDY10 is provided in Figure 2.6. *R. bovis* ASCUSDY10 is [redacted] (b) (4). A working cell culture stock is maintained by Native Microbials and used for the seed fermentation. [redacted] (b) (4) Details on the manufacturing process are provided in Appendix 010 (CONFIDENTIAL).

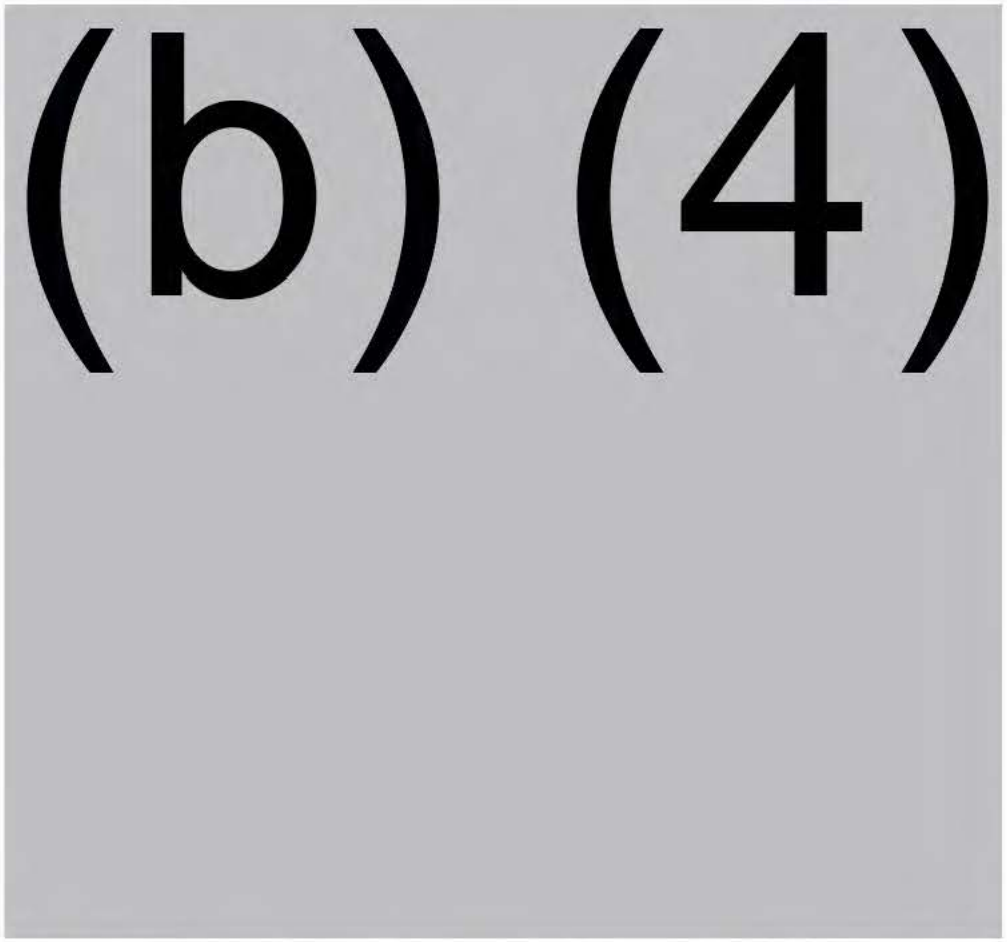

Figure 2.6 Schematic Overview of the Manufacturing Process

2.3.3 Production Controls

Commercial manufacture of *R. bovis* ASCUSDY10 will be in accordance with current Good Manufacturing Practices (cGMP) and a Food Safety Plan is in place as required by 21 CFR §507. The requirements of the FSMA Preventive Controls (per §507) will be applied at all stages of the production, processing and distribution.

2.4 Product Specifications and Batch Analyses

2.4.1 Proposed Product Specifications for Post-fermentation

Appropriate feed-grade specifications have been established for *R. bovis* ASCUSDY10 after fermentation and are presented in Table 2.23. Although the notified substance does not encode genes to produce botulinum toxins, nor does it express any such toxins, the fermentation is anaerobic and therefore susceptible to contamination by anaerobes. Out of an abundance of caution, botulinum toxins are tested (FDA BAM mouse method) to assure the batch is free from contamination by botulinum toxin-producing microbes. Copies of the methods of analysis are provided in Appendix 007.

Table 2.23: *R. bovis* ASCUSDY10 Post-fermentation Specifications		
Botulinum toxins	(b) (4)	FDA BAM

Abbreviations: BAM = Bacteriological Analytical Manual

2.4.2 Batch Analyses for Post-fermentation

Three batches of *R. bovis* ASCUSDY10 post-fermentation representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.24 and the Certificates of Analysis are provided in Appendix 013. No botulinum toxins were identified in any of the batches (Appendix 008).

Table 2.24: Analytical Results for 3 Batches of *R. bovis* ASCUSDY10 Post-fermentation			
			Analytical Results
Parameter	Unit	Specification	(b) (6)
Botulinum toxins*	Per 2 g	Negative	(b) (6)

* Testing done at end of fermentation process, prior to centrifugation

2.4.3 Proposed Product Specifications for the *R. bovis* ASCUSDY10 Freeze-dried Powder

Appropriate feed-grade specifications have been established for *R. bovis* ASCUSDY10 manufactured as a freeze-dried powder and are presented in Table 2.25. Copies of the methods of analysis are provided in Appendix 012.

Table 2.25: *R. bovis* ASCUSDY10 Freeze Dried Powder Specifications		
Parameter	Specification Limits	Analytical Method
Viable cell count	(b) (6)	Internal Method

Abbreviations: CFU = colony forming units. Internal Method Appendix 012C

2.4.4 Batch Analyses for *R. bovis* ASCUSDY10 Freeze-dried Powder (FDP)

Three batches of *R. bovis* ASCUSDY10 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.26 and the Certificates of Analysis are provided in Appendixes 013A-C.

Table 2.26: Analytical Results for 3 Batches of *R. bovis* ASCUSDY10 FDP			
Parameter	Unit	Specification	Analytical Results
			(b) (4)
Viable cell count	CFU/g	> 1 x 10^9 CFU/g	(b) (4)

Abbreviations: CFU = colony forming units.

2.4.5 Proposed Product Specifications for the *R. bovis* ASCUSDY10 Fat Encapsulate

Appropriate feed-grade specifications have been established for *R. bovis* ASCUSDY10 manufactured as a fat encapsulate and are presented in Table 2.27. Copies of the methods of analysis are provided in Appendices 007 and 012.

Table 2.27: *R. bovis* ASCUSDY10 Fat Encapsulate Product Specifications		
Parameter	Specification Limits	Analytical Method
Viable cell count	(b) (4)	Internal Method
Coliform		AOAC 2018.13
E. coli		AOAC 2018.13
Salmonella		AOAC 2013.01
Listeria		AOAC 2013.10

Abbreviations: CFU = colony forming units; BAM = Bacteriological Analytical Manual; AOAC = Association of Official Analytical Chemists. Internal Method Appendix 12C

2.4.6 Batch Analyses for *R. bovis* ASCUSDY10 Fat Encapsulate

Three batches of *R. bovis* ASCUSDY10 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.28 and the Certificates of Analysis are provided in Appendixes 013D-F.

Table 2.28: Analytical Results for 3 Batches of *R. bovis* ASCUSDY10 Fat Encapsulate			
Parameter	Unit	Specification	Analytical Results
			(b) (4)
Viable cell count	CFU/g	> 2 x 10^7 CFU/g	(b) (4)
Coliform	CFU/g	<10	
E. coli	CFU/g	<10	
Salmonella	Per 25 g	Negative	
Listeria	Per 25 g	Negative	

Abbreviations: CFU = colony forming units.

2.4.7 Additional Analytical Data

The levels of heavy metals are also periodically monitored in batches of *R. bovis* ASCUSDY10. Three batches of *R. bovis* ASCUSDY10 representative of the commercial material were analyzed to verify that the levels of these contaminants fall within acceptable ranges. The results are summarized in Table 2.29 and the Certificates of Analysis from analytical laboratories are provided in Appendix 014. On the basis of the analytical data, no specifications for heavy metals are considered necessary. Based on the level of use, there is no need to identify a specification on these heavy metals based on their insignificant levels and a safety assessment as provided in Part 6.

Table 2.29: Further Analytical Results for 3 Batches of *R. bovis* ASCUSDY10			
Parameter	Unit	Analytical Results	Analytical Method
		(b) (4)	
Arsenic	ppm	(b) (4)	AOAC 2015.01
Cadmium	ppm		AOAC 2015.01
Lead	ppm		AOAC 2015.01
Mercury	ppm		AOAC 2015.01

Abbreviations: AOAC = Association of Official Analytical Chemists. ND= None Detected

2.5 Scale Up and Commercial Size Batches

Scale up efforts have demonstrated that larger batches can be made using the same process and media as described in Appendix 010 (CONFIDENTIAL) for the manufacturing of the notified substance without effect on specifications. Batch sizes of the commercial size lots in comparison to the research/pilot-size batches used for testing within the dossier are presented in Appendix 016Y (CONFIDENTIAL). Testing done at the various stages toward the final encapsulated organism at full scale are shown together in Tables 2.30 – 2.35, indicating performance against the specifications established during development.

2.5.1 Research/Pilot Batch Sizes Compared to Commercial-Size Batches

Batches used in the establishment of the specifications and for presentation in the GRAS Dossier are represented at the following sizes at the various stages of manufacturing in Appendix 016Y. The three independent batches of fat encapsulated *R. bovis* ASCUSDY10 were produced in a manner consistent with manufacturing scale relevance. (b) (4) Processes employed mimic manufacturing scale industrial norms in fermentation technology such that key performance and quality attributes at scale were expected to meet or exceed those achieved with the three pilot scale batches. (b) (4)

As a result of the scale-relevant equipment used for processing, the 3 lots presented for the indicated substance were intended to reflect the anticipated viable cell count, microbial contaminants, heavy metal contents, and stability of the commercial products of that same indicated substance (*R. bovis* ASCUSDY10).

2.5.2 Commercial Size Testing

To confirm that the scale-relevant equipment were representative of full-size batch production, three lots produced in (b) (4)-liter fermentors were created and tested according to proposed specifications. Details on batch sizes and yields at the various stages are contained in Appendix 016Y.

Tables 2.30 through 2.32 represent test results at the various stages from three commercial-size batches. Certificates of Analysis and Test Reports are found in Appendixes 016A-016X.

Table 2.30: Analytical Results for Three (b) (4) L Batches of *R. bovis* ASCUSDY10			
Parameter	Unit	Specification	Analytical Results (b) (4)
Viable cells count	CFU/g	≥1.0 x 10^{10}	(b) (4)
Coliforms	CFU/g	<10	
E. coli	CFU/g	<10	
Salmonella	Per 25 g	Negative	
Listeria	Per 25 g	Negative	
Botulinum toxins	Per 2 g	Negative	

Table 2.31: Analytical Results for Three Freeze-Dried Batches of *R. bovis* ASCUSDY10			
Parameter	Unit	Specification	Analytical Results (b) (4)
Viable cells count	CFU/g	≥1.0 x 10^{9}	(b) (4)

Table 2.32: Analytical Results for Three Fat Encapsulated Batches of *R. bovis* ASCUSDY10			
Parameter	Unit	Specification	Analytical Results (b) (4)
Viable cells count	CFU/g	≥2.0 x 10^{7}	(b) (4)
Coliforms	CFU/g	<10	
E. coli	CFU/g	<10	
Salmonella	Per 25 g	Negative	
Listeria	Per 25 g	Negative	

2.5.3 Heavy Metal Testing on Commercial Size Batches

The following Table (2.33) represents heavy metals testing on the Commercial size batches represented in Section 2.4.2. Certificates of Analysis are found in Appendix 016. Although heavy metals are not required to be tested for each lot at various stages in manufacturing, additional testing was done on the three commercial-size lots previously described to show scalability.

Table 2.33:	Heavy Metal Results for Commercial Batches of R.bovis ASCUSDY10 at the Fermentation and Finished Fat Encapsulate Stages						
		Fermentation			Fat Encapsulate		
Test	Unit	Lot	Lot	Lot	Lot	Lot	Lot
Arsenic	ppm	(b) (4)					
Cadmium	ppm						
Lead	ppm						
Mercury	ppm						

ND= None Detected (LOD/LOQ ppm: As=0.004/0.016; Cd= (b) (4); Hg (b) (4); Pb (b) (4))

2.5.4 Commercial Scale-Up Summary

It can be seen from the testing of the three (b) (4)-liter batches that the scaled-up organism was comparable in all testing to the smaller scale fermentations used in the test batches and that fermentation parameters and ingredients transferred well to full size operations. As seen in testing, there was no further addition of metals by doing fermentation in large-size stainless steel fermenters versus lab-scale models.

2.6 Stability

2.6.1 Shelf-Life Stability Data

Native Microbials guarantees conformity of fat encapsulated *R. bovis* ASCUSDY10 to the product specification (see Table 2.22) for a minimum of 12 months when stored in the original, unopened packaging at refrigerated temperature (2 - 10°C). The proposed shelf life is supported through ambient (5°C) and accelerated (25°C) stability studies in which 3 batches of fat encapsulated *R. bovis* ASCUSDY10 representative of the commercial material were stored at 5°C and 25°C, respectively for 12 months. Packaging was done using the same materials as provided in Appendix 06.

2.6.1.1 Stability Study at 5°C

The results of the stability study conducted at 5°C for 12 months on *R. bovis* ASCUSDY10 are summarized in Table 2.34 and the report is provided in Appendix 015A. Over the period evaluated, few changes in the viable cell count were observed representing stable viability for 12 months at refrigerated (ambient) temperatures (shown in Figure 2.7) for the 3 batches of *R. bovis* ASCUSDY10.

Table 2.34: Results of a Stability Study on 3 Batches of *R. bovis* ASCUSDY10 Stored at 5°C			
	(b) (4)		
Months	Viable Count (cfu/g)	Viable Count (cfu/g)	Viable Count (cfu/g)
0	(b) (4)		
1			
2			
3			
6			
9			
12			

Abbreviations: CFU = colony forming units; SD = standard deviation.

2.6.1.2 Stability Study at 25°C

The results of the stability study conducted at 25°C for 12 months with *R. bovis* ASCUSDY10 are summarized in Table 2.35 and the report is provided in Appendix 015B. Over the period evaluated only minor changes (less than one log) in the viable cell count were observed representing a decay rate plotted in Figure 2.7 for the 3 batches of *R. bovis* ASCUSDY10.

Table 2.35: Results of a Stability Study on 3 Batches of *R. bovis* ASCUSDY10 Stored at 25°C			
	(b) (4)		
Months	Viable Count (cfu/g)	Viable Count (cfu/g)	Viable Count (cfu/g)
0			
1			
2			
3			
6			
9			
12			

(b) (4)

Abbreviations: CFU = colony forming units; SD = standard deviation.

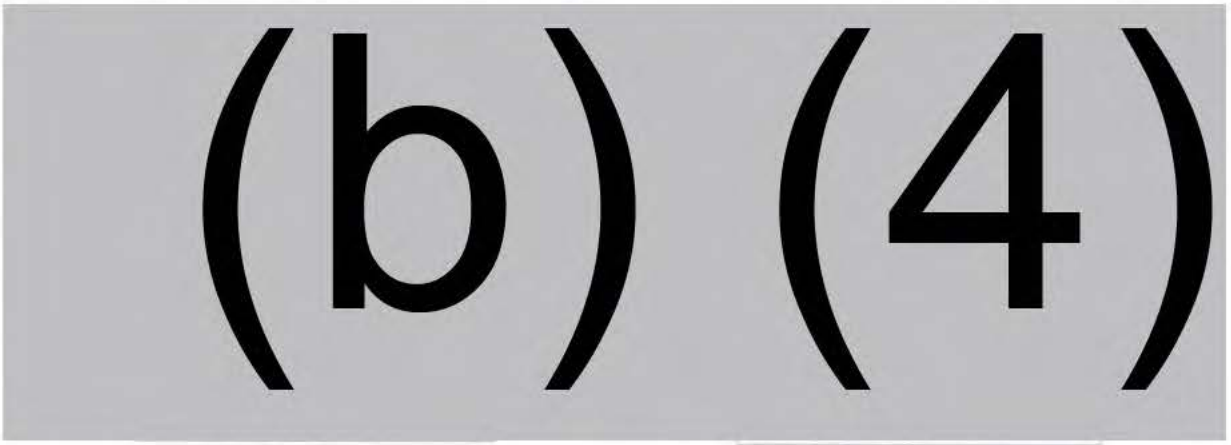

Figure 2.7 Rates of Decay at 5°C and 25°C. Log_{10} CFU/g measurements are plotted, with the minimum specification represented as zero on the y-axis. Shaded area represents the 95% confidence interval.

2.6.1.3 Shelf Life Prediction

Rates of decay for each lot at each temperature show that, for both accelerated and ambient conditions, at no time were the three lots tests found to lose viability below minimum specification and show less than one log loss over the year, confirming the assigned one year shelf life at ambient (5°C) temperatures. Because storage at higher temperatures (25°C) also showed good stability, excursions outside the

refrigerator could be tolerated for reasonable periods of time without affecting the viability of the organism.

2.6.2 In-Feed Stability

As mentioned in Part 1, *R. bovis* ASCUSDY10 may be incorporated into the diet of dairy cattle as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The strain is encapsulated with fat to generate a stable product suitable for handling under practical commercial farming conditions in the U.S. The dry matter intake of dairy cattle is optimized by feeding fresh TMR on a twice daily basis. The forage content is typically adjusted to meet the nutrient requirements of the animals on a pen basis. Under the conditions of intended use, *R. bovis* ASCUSDY10 may be mixed directly into the TMR or added as a top-dressing at the point of use. On this basis, long-term stability is not relevant, and an in-feed stability study was not conducted.

2.6.3 Homogeneity Data

Due to the highly similar manufacturing process and ensuing encapsulated cell size, the powder attributes, formula, particle size and moisture content (see Appendix 11) of the commercial offering of *R. bovis* ASCUSDY10 was noted to be nearly identical to that described in a recent prior submission (AGRN 42, *Butryrivibrio fibrisolvens*) and therefore a separate homogeneity study was deemed unnecessary.

2.6.4 Manufacturing Summary

Native Microbials will manufacture a safe stable product for dairy cattle meeting cGMP and FSMA compliance. This was demonstrated through batches of product meeting product specifications for contaminants, heavy metals and potency. The product is packaged in moisture protected barrier bags and has been shown to be stable under normal storage (refrigerated) conditions.

2.7 Effect of the Notified Substance

This portion of the notice addresses the requirements specified in 21 CFR 570.230(d):

> (d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

The GRAS Final Rule (81 FR 54960) provides interpretation of this regulation specific to animal feed ingredients in response to comment 144: "We agree that data and information bearing on the physical or other technical effect the notified substance is intended to produce are only necessary when they bear on safety." A product like phytase would require data, however, the intended purpose of supplementation of *R. bovis* ASCUSDY10 is to support normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *R. bovis* ASCUSDY10 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. Thus, data and information demonstrating the intended effect of *R. bovis* ASCUSDY10 in the feed of dairy cattle are not required as part of this GRAS notice.

As a commensal microorganism, feeding *R. bovis* ASCUSDY10 would supplement the existing *R.bovis* population in the rumen microbiome. The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour *et al.*, 2015), and is further described below in context of technical effect and animal safety (Part 6 of this notice). Should *R. bovis* not act to support the digestion of carbohydrates such as glucose and starch, there would be no safety impact, as the existing rumen microbiome will continue to ferment feed, and the feed was formulated to assure nutrient requirements were met without consideration of the potential for increased digestion of carbohydrates.

2.7.1 Rumen Microbiome

The rumen microbiome is highly variable depending on several factors including age, breed, diet composition, time after feeding, season, stage of lactation, location, and farm management practices (Pitta *et al.*, 2016; Furman *et al.*, 2020; Henderson *et al.*, 2015; Wallace *et al.*, 2019). Diet, in particular, has been shown to be the main driver of microbiome composition (Johnson and Johnson, 1995; Brulc *et al.*, 2009; Carberry *et al.*, 2014; Ghaffari *et al.*, 2014; Kumar *et al.*, 2015; Deusch *et al.*, 2017; Mizrahi and Jami 2018; Belanche *et al.*, 2019). To better understand the microbiome in context of this variability, many studies have focused on identifying and characterizing the core rumen microbiome, which is the common assemblage of microorganisms that are characteristic of the rumen environment (Petri *et al.*, 2013; Xue *et al.*, 2018; Henderson *et al.*, 2015; Wallace *et al.*, 2019; Furman *et al.*, 2020; Kumar *et al.*, 2015; Jami *et al.*, 2013; Kittlemann *et al.*, 2013; Lima *et al.*, 2015; Fouts *et al.*, 2012).

Published literature has identified a core rumen microbiome that is primarily dominated by bacteria phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Fibrobacteres*. This set of microbial taxa provide the basal level of fermentation required for dairy cow nutrition regardless of animal management and diet (Jami and Mizrahi, 2012; Henderson *et al.*, 2015; Lima *et al.*, 2015; Deusch *et al*, 2017; Huws *et al.*, 2018; Xue *et al.*, 2018). To better assess the impact of *R. bovis* ASCUSDY10 on the existing rumen microbiome, datasets from published and internal microbiome studies were compiled and analyzed to identify the typical abundance of core rumen microbiome members in dairy cows (Appendix 018). If the abundances of core microbiome members are within typically observed ranges, it is reasonable to conclude that rumen fermentation is also operating within normal ranges. Rumen microbiome datasets collected from *in vivo* studies feeding *R. bovis* ASCUSDY10 were analyzed to corroborate that no large shifts in the core microbiome occurred (Appendix 018). Thus, no detrimental effects of rumen fermentation are expected from supplementing *R. bovis* ASCUSDY10 in feed.

2.7.2 Impact of Failure of the Notified Substance

Feeding *R. bovis* ASCUSDY10 to dairy cattle supports the existing populations of *R. bovis* in the rumen to support ruminal feed fermentation with no alterations to the standard feeding regime. The diet offered to the dairy cows would be formulated to meet the existing nutritional needs of the animal (NRC, 2001). Therefore, the effect of the notified substance is not required for the general well-being and normal performance of dairy cattle. Should *R. bovis* ASCUSDY10 fail, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Further, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. Any non-performing *R. bovis* ASCUSDY10 or deceased *R. bovis* ASCUSDY10 would pass through the GI tract with

the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016). Therefore, the "failure" of *R. bovis* ASCUSDY10's intended use would not raise a safety concern. General recognition of this assertion has been demonstrated in the literature.

Several published experiments have directly investigated the impacts of DFMs by comparing groups of animals receiving a "dead" microbial against a variety of treatment conditions. Cunha, *et al.* (2019) compared heifers fed a basal diet against heifers fed the same basal diet containing a live yeast or inactive yeast supplement (2 different doses) in a 5x5 Latin square experimental design with 15-day periods. Live and dead yeasts were administered to the appropriate animals after each feeding through infusion directly into the rumen. No differences in digestibility were observed between the control, live yeast, or either of the inactive yeast doses. No differences were observed in feed intake nor animal behavior. Hence the inactive yeast did not alter the overall digestion of the feed, nor impact the health of the animals. Feeding inactive yeast did not decrease rumen function.

Muscato, *et al.* (2002) evaluated the feeding of fresh and inactivated rumen fluid to calves in a series of four experiments. The animals were dosed daily with 8 mL of either fresh or inactivated rumen fluid obtained from a cannulated Holstein cow from 0-6 weeks of age. In the first experiment, calves were either fed a typical basal ration or the same basal ration supplemented with fresh rumen fluid. In the second experiment, calves were fed the basal ration with either the cell pellet of fresh rumen fluid, supernatant of fresh rumen fluid, or no addition. In the third experiment, calves were fed a basal ration, or a basal ration supplemented with autoclaved rumen fluid. Autoclaving rumen fluid ensures microbial death, thus inactivating the biological component. The fourth experiment had a similar set-up to the third experiment, but rumen fluid was only fed for 5 days rather than 6 weeks. In the studies that evaluated autoclaved rumen fluid, the number of days of scouring were significantly decreased compared to the control. Similarly, the calves receiving autoclaved rumen fluid experienced higher gains in the first two weeks, but by the end of the experimental period there was no impact on growth. There were no differences in the outcomes of calves receiving fresh rumen fluid as compared to calves receiving autoclaved rumen fluid. This study suggests that the feeding of inactivated microorganisms does not decrease rumen function or create a safety concern when fed to animals.

Philippeau, *et al.* (2017) fed multiple DFM treatments to investigate the effects of DFM on rumen fermentation characteristics and digestibility. Animals were assigned one of four treatment groups: control (CON), *Propionibacterium* P63 (P63), *Propionibacterium* P63 and *Lactobacillus plantarum* 115 (P63+Lp), or *Propionibacterium* P63 and *Lactobacillus rhamnosus* 32 (P63+Lr). Each strain was administered at 10^{10} cfu/d. No change in ruminal VFA concentration was observed, and only P63 was found to impact the concentration of some milk fatty acids. pH increased on average 0.18 units in all DFM groups as compared to the control. Although the study did not demonstrate the positive response in performance as was expected, there was no negative change in the assessed parameters that may suggest a decrease in health. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight *et al.*, 2007, Abu-Tarboush *et al.*, 1996, Higginbotham *and Bath.*, 1993, McGilliard and Stallings, 1997). In Weiss *et al.* (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al.* (2008) fed *Prevotella bryantii* 25A to dairy cows

in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al.* (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman *et al.*, 2011; Hagg *et al.*, 2010, Zebeli *et al.*, 2012; Hagg, 2007; Kung and Hession, 1995). A *Lactobacillus*-based probiotic fed alone and in combination with *S. cerevisiae* showed no change in milk production or efficiency in early-lactation dairy cows (Boga and Gorgulu, 2007). In a meta-analysis conducted at INRA, 33 probiotic bacteria studies with or without yeast were evaluated for their impact on the production and health of dairy and beef cattle (Lettat et al., 2012). Variable performance and rumen impacts were observed, however the study indicated no negative health consequences were reported. In the studies summarized above, even though the direct fed microbials did not achieve the performance response expected, there was no indication of a safety concern.

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to the fermentation characteristics of the rumen or animal well-being. In the case of *R. bovis* ASCUSDY10, if the DFM failed to provide improved digestibility, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Since no alterations are made to the standard feeding regime when using this product, the value of the feed that would be digested and utilized for the nutrients required to sustain life is identical between the control and treated group. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001). Any non-performing *R. bovis* ASCUSDY10 or deceased *R. bovis* ASCUSDY10 would pass through the GI tract with the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016).

In this respect, based on the results of published comparative studies, *R. bovis* ASCUSDY10 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, and as stated previously, while *R. bovis* ASCUSDY10 may aid the digestion of feed, the effect is not required for the well-being of dairy cattle. Thus, the absence of the anticipated effect of *R. bovis* ASCUSDY10 on feed digestion by dairy cattle would not have an impact on safety. Native Microbials' product labeling would not suggest a change in normal feeding regime, and its use would be specific for gaining additional nutritional value from a typical balanced ration. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001).

2.7.3 Summary

In summary, the intended use of *R. bovis* ASCUSDY10 is to support ruminal feed digestion. The rumen microbiome naturally varies and is greatly influenced by diet. The supplementation of *R. bovis* ASCUSDY10 does not alter the core rumen microbiome beyond its natural range. No detrimental effects of rumen fermentation are expected from supplementing *R. bovis* ASCUSDY10 in feed.

Further, it is Native Microbials' understanding that the regulatory hurdle provided in §570.230(d), is not applicable to the conclusion of the generally recognized as safe substance *R. bovis* ASCUSDY10. Dairy cows would receive regular feed that have been formulated to meet the nutritional requirements of the animal.

The "failure" of *R. bovis* ASCUSDY10's intended use would not raise a safety concern, as the animals continue to receive the requirement nutritional needs from their regular feed. Therefore, there is no regulatory requirement to provide specific utility data to support the intended use.

PART 3 – TARGET ANIMAL AND HUMAN EXPOSURE

3.1 Target Animal Exposure

3.1.1 Exposure to the Direct Fed Microbial Strain

As mentioned in Part 1, *R. bovis* ASCUSDY10 is intended for use as a source of viable microorganisms in feed for dairy cattle. The microbial strain will be delivered as a fat encapsulated direct fed microbial to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *R. bovis* ASCUSDY10 may be incorporated into the diet of dairy cattle include as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The product will be incorporated into dairy cattle feed at the recommended use level of $1x10^8$ CFU of *R. bovis* ASCUSDY10/cow/day. As mentioned in Part 2.3, the fat encapsulated product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried R. *bovis* ASCUSDY10 powder. Thus, under the conditions of intended use, dairy cattle will be exposed to a maximum of 1 g of the unencapsulated *R. bovis* ASCUSDY10 per day.

3.1.2 Exposure to the Other Components of the Fat Encapsulated Product

At the intended intake of $1x10^8$ CFU *R. bovis* ASCUSDY10/cow/day, the animal will be exposed to up to 5 g of the notified substance at a min. $2x10^7$ CFU/g titer. The product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried *R. bovis* ASCUSDY10 powder (see Appendix 010). As mentioned in Part 2, the amount of hydrogenated glycerides, sodium sulfate, and freeze-dried *R. bovis* ASCUSDY10 powder is adjusted for each batch to standardize the viable cell count. These encapsulation ingredients are acceptable for use in dairy cattle feed and comply with the corresponding ingredient definitions in the AAFCO Official Publication (AAFCO 2023; ingredient definitions 73.311 and 57.109 - see Appendix 010). Under these conditions of use, the animal will be exposed to a maximum of 2.5 g of hydrogenated glycerides and 1.5 g of sodium sulfate. Considering that the typical dry matter intake by the dairy cattle is about 25 kg/cow/day, the contribution of hydrogenated glycerides to the dairy ration is expected to be no more than 0.006% DM. While the fat concentration of a typical dairy diet is reported to be relatively low (approximately 2.5% DM), supplemental fats can be added to achieve a total ration content of around 6% DM (MSD Veterinary Manual (online), 2023). On this basis, the use of hydrogenated glycerides or similar acceptable fat source as an encapsulating aid in the manufacture of fat encapsulated *R. bovis* ASCUSDY10 will have a negligible impact on the total fat intake by dairy cattle under the conditions of use. Similarly, an intake of 1 g/cow/day of sodium sulfate will provide dairy cattle with approximately 0.48 g of sodium/cow/day, representing less than 0.004% of the DM intake. The maximum tolerable levels of sodium chloride set by the National Research Council (NRC) for lactating cows is 3% of DM intake, equivalent to around 1% DM of sodium. Thus, the use of sodium sulfate as an encapsulating agent in the manufacture of fat encapsulated *R. bovis* ASCUSDY10 is not expected to have any significant impact on the overall sodium intake by dairy cattle under the intended conditions of use. Another element of interest is sulfur. The use of *R. bovis* ASCUSDY10 would provide approximately 1 g of sodium sulfate or 0.34 g of sulfur per day. The NRC (2016) has suggested that Total Mixed rations (grain

based) of cattle diets should be at a maximum tolerable level of 0.3% sulfur (75 g/cow/day), as such this ingredient would provide an insignificant amount of the total sulfur in the diet of the dairy cow.

3.1.3 Background Exposure to the Microorganism

As mentioned in Part 2.1, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and in this respect, *R. bovis* ASCUSDY10 will contribute to the native population of *Ruminococcus* species in the gut of the animal (see Appendix 018). *Ruminococcus* species, including *Ruminococcus bovis,* are part of the rumen microflora and is routinely isolated from livestock feces and rumen content (Boonsaen et al. 2017; Boonsaen et al. 2019; Chassard et al. 2012; Domingo et al. 2008; Ezaki 2015; Flint et al. 2008; Holdeman and Moore 1974; Klieve et al. 2007; Leitch et al. 2007; Stewart et al. 1997). Thus, while not present to a significant or intentional degree in feedstocks, background exposure by dairy cattle to *R. bovis* from the environment is likely to be significant.

3.2 Human Exposure

R. bovis ASCUSDY10 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. As mentioned in Part 2.1, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow. *R. bovis* ASCUSDY10 is regularly identified in the rumen and fecal content of various ruminants, including dairy cows, beef cattle, and sheep, across the globe (abundance ranges from 1.9E-05% to 35%), suggesting its presence is common and prevalent in ruminants (Appendix 018). Thus, any potential human exposure of *R. bovis* already occurs naturally in the ruminant livestock industry.

We also conducted a thorough Google scholar search using various combination of search-terms, including "pathogen", "safety", "infection", and "disease", to determine the safety of *R. bovis* according to the scientific community (Appendix 017). Because the publications on *R. bovis* is limited due to the species' recent naming, the search was also conducted using genus name "*Ruminococcus*". As stated in Part 2.2.2, *Mediterraneibacter* used to be named *Ruminococcus* despite its distant phylogenetic lineage. Further review of the returned searches revealed that all pathogen/disease related results were related to species of *Mediterraneibacter*. No pathogenicity nor other safety concerns have been reported for *Ruminococcus* of the *Oscillospiraceae* lineage (Appendix 017). This corroborates our *in vitro* and *in silico* analyses of *R. bovis* ASCUSDY10 that no pathogenicity, virulence, or toxicity concerns are associated with the notified microorganism with its intended use (Parts 2.2.6-2.2.7).

3.2.1 Withdrawal Period After Use of Notified Substance

No withdrawal period is considered necessary between animal use of the notified substance and human use of the milk on the basis that *R. bovis* ASCUSDY10 is native to the rumen of dairy cattle and, as detailed in Parts 2 and 6, the strain has been shown to have no pathogenic or toxigenic properties for humans.

PART 4 – SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *R. bovis* ASCUSDY10 .

PART 5 – EVIDENCE BASED ON COMMON USE BEFORE 1958

Not applicable.

PART 6 – NARRATIVE

6.1 Basis of GRAS Conclusion

Phenotypic characterization and literature have historically been the primary source for assessing the safety of Direct Fed Microbials (DFMs). With the recent advancements in sequencing technologies, it has become possible to obtain precise whole genome sequences of specific strains. This progress, coupled with the rapid expansion of sequencing databases that include detailed annotations of genes linked to pathogenicity, virulence factors, and antimicrobial synthesis, has significantly enhanced the accuracy of bacterial strain safety evaluations. Today, a comprehensive assessment of the safety of microorganisms involves not only the in-depth analysis of whole genome sequences but also the integration of relevant *in vitro* data. This holistic approach ensures a more thorough and accurate substantiation of the safety of specific microbial strains.

The notified substance, *R. bovis* ASCUSDY10, is the type strain of *Ruminococcus bovis*, a species within the *Oscillospiraceae* family (Gaffney et al., 2022). The taxonomic classification was determined via both 16S rRNA gene sequencing/phylogeny and whole genome comparison (Section 2.2.2). It is important to mention that *Mediterraneibacter*, a genus within the *Lachnospiraceae* family, was formerly named as *Ruminococcus* (Togo et al., 2018; Oren et al., 2019; personal communication with Dr. Oren). To reduce confusion, subsequent references in the text to *"Ruminococcus"* refers to *Ruminococcus* of the *Oscillospiraceae* family, while *"Mediterraneibacter"* refers to *Ruminococcus* of the *Lachnospiraceae* family.

Native Microbials has provided current scientific rigor that supports the GRAS conclusion of *R. bovis* ASCUSDY10, summarized as follows:

1. *R. bovis* ASCUSDY10 is unambiguously identified as a member of *Ruminococcus* of the *Oscillaspiraceae* lineage. No virulence or pathogenicity to humans or animals have been associated with *R. bovis* and *Ruminococcus* of the *Oscillospiraceae* lineage to date in literature (Appendix 017). Corroborating this, genomic interrogation revealed no confirmed virulence, pathogenic, toxicity, or pro-inflammatory genes in *R. bovis* ASCUSDY10 (Part 2.2.6). Although low threshold presence of potential hemolysis genes were detected, rigorous *in vitro* testing confirmed the lack of phenotypic expression (Appendix 021).
2. *R. bovis* is ubiquitously present in the rumen of various ruminant species across the globe. Among 628 lactating Holstein and Jersey dairy cows evaluated by Native Microbials and Native Microbials sponsored independent research studies, *R. bovis* was naturally present in the rumen microbiome of all animals (0.001% - 16% in abundance; Appendix 018). In addition, 19 independent studies with sequencing data were queried from Google Scholar. These studies comprised a total of 14,637 samples from 1,931 ruminants (dairy cows, beef cattle, sheep, and buffalo) across 12 countries. *R. bovis* was detected in 14,616 samples (99.9%) with abundance up to 35% (Appendix 018).
3. *R. bovis* ASCUSDY10 has been fed to more than 250 lactating dairy cows in-feed daily for 16 to 39 weeks (Dickerson et al., 2022; Valldecabres et al., 2022; Goldsmith et al., 2023; (b) (4)

unpublished data, (b) (4) unpublished data; (b) (4) unpublished data). No adverse health effects were observed (Appendix 019).

Based on our detailed understanding of the impact of feeding *R. bovis* ASCUSDY10 in dairy cattle, Native Microbials has met the standard of safety "that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use."

6.1.2 *Ruminococcus* and *R. bovis* are Common and Ubiquitous in Ruminants, Including Lactating Dairy Cows.

As discussed in Section 2.7, commensal rumen microorganisms are essential for maintaining health and nutrition in ruminants. Leveraging next-generation sequencing technologies, the scientific community has identified the genus *Ruminococcus* and its member *R. bovis* as rumen commensal microorganisms.

The following studies specifically identified the genus *Ruminococcus* as part of the core microbiome in ruminants, including dairy cows:

Xue et al. (2020): The authors studied the rumen microbiome structure of 334 dairy cows under the same management at various stages of lactation. The study found that *Ruminococcus* is consistently present despite differences in milk yield and lactation stage. While there are variations in *Ruminococcus* abundance among individual cows, on average, *Ruminococcus* comprises 1.5 ± 0.2% of the core rumen microbiome in lactating dairy cows.

Petri et al. (2013): The authors investigated dietary impact on microbiome structure, highlighting *Ruminococcus* spp. and *Fibrobacter succinogenes* as substantial members of the rumen microbial communities of Angus heifers on mixed forage diets.

Global Rumen Census (Henderson et al., 2015): The authors conducted a comprehensive survey across 32 ruminant species (including dairy cows) from 35 countries, identifying *Prevotella*, *Butyrivibrio*, and *Ruminococcus* as the dominant bacterial genera in the rumen. *Ruminococcus* was present in all surveyed ruminants and comprised 3.6% of the rumen microbial community.

AlZahal et al. (2017): The authors observed that the *Oscillospiraceae* (*Ruminococcaceae*) family accounted for approximately 7% of the rumen microbial community in lactating dairy cows fed high-forage diets, particularly associating with solid fibers.

Wirth et al. (2018): The authors studied the rumen fluid microbial communities of Holstein dairy cows, finding members of the *Ruminococcus* genus (~35%) to be the second most prevalent group within the rumen core microbiome.

Seshadri et al. (2018): The authors described **the Hungate1000 project**, which isolated 410 unique bacteria and archaea from rumen content of sheep, beef cattle, goat, moose and dairy cows, with many belonging to the *Ruminococcus* species, emphasizing its prevalence and diversity in the rumen. **The Hungate1000 project** yielded 21 unique *Ruminococcus* strains from healthy cow rumen contents, underlining the ubiquity of this group in the rumen environment.

Literature also showed that members of *Ruminococcus* perform a wide array of beneficial enzymatic functions on feed. *Ruminococcus* species encompass cellulolytic bacteria such as *R. flavefaciens*, *R. albus*,

R. callidus, *R. bicirculans*, and *R. champanellensis*, along with the amylolytic bacterium *R. bromii* and *R. bovis*, showing adaptability across ruminant and monogastric hosts and emphasizing their essential roles in feed/food digestion across species. Do *et al.* (2018) identified *R. bicirculans* cellulase genes in the rumen of healthy goats. Dai *et al.* (2015) demonstrated that 14.7% of the hemicellulases and 16.1% of the cellulases encoding genes were contributed by *R. flavefaciens*, while 7.8% of the hemicellulase and 13.6% cellulase encoding genes were like those of *R. albus*. *R. bromii* is equipped with a diverse group of efficient amylase enzymes, which are instrumental in breaking down resistant starch that are otherwise indigestible in humans (Ze et al., 2012). *R. bovis*, a common rumen microorganism, has the similar amylase enzymes that are found in *R. bromii*, suggesting its unique role in assisting in ruminal feed digestion of resistant starch (Gaffney et al., 2021).

R. bovis is also prevalent in lactating dairy cows. However, it was frequently grouped with unclassified bacteria because it was unnamed until recently (Gaffney et al., 2021). Leveraging the next-generation sequencing technologies, we were able to identify *R. bovis* and determine its abundance based on its 16S rRNA gene identity (98.5%) from both internal and external studies (Meier-Kolthoff et al., 2013; Kim et al., 2014):

Native Microbials Rumen Microbiome Surveys: Native Microbials surveyed the rumen microbiome of lactating dairy cows across the US consuming different diets and *R. bovis* was detected in all animals with an abundance ranging from 0.001% to 13% (Appendix 018).

Independent Rumen Microbiome Surveys: In another 6 independently conducted experiments sponsored by Native Microbials (Appendices 019A-F; Valldecabres et al., 2022; Goldsmith et al., 2023), a DFM containing *R. bovis* ASCUSDY10 was administered to 263 lactating dairy cows in feed daily, while 365 lactating dairy cows did not receive *R. bovis* ASCUSDY10. *R. bovis* was naturally present and represented 0.16%-15% of the rumen bacterial community in all animals that did not receive *R. bovis* ASCUSDY10, indicating the *R. bovis* is common and prevalent in the dairy cow rumen (Appendix 018).

***R.bovis* Prevalence from Literature:** Native Microbials identified 19 previously published studies that surveyed rumen samples with publicly available rumen microbiome sequencing data and well-documented metadata (Nelson et al., 2014; Myer et al., 2016; Wetzels et al., 2016; Deusch et al., 2017; Kamke et al., 2017; Scharen et al., 2017; van Lingen et al., 2017; Wetzels et al., 2017; Biscarini et al., 2018; Chiariotti et al., 2018; Difford et al., 2018; Neubauer et al., 2018; López-García et al., 2018; Wetzels et al., 2018; Wallace et al., 2019). These studies included samples from various breeds of dairy cows, beef feedlot cattle, sheep, and buffaloes around the globe (12 countries and 1,931 animals). *R. bovis* was detected in 99.9% of the sequence files (14,616 out of 14,637). The exact number of animals containing *R. bovis* could not be determined because the studies did not share the mapping file between individual animals and sequence IDs. Among the 14,616 sequence files from which *R. bovis* were identified, the abundance of *R. bovis* ranged from 1.9E-5% to 35% (Appendix 018).

Together these findings reaffirm the prevalence of *Ruminococcus* and *R. bovis* in ruminants, including dairy cows, and its involvement in supporting rumen feed digestion. In addition, no potential threats posed by *Ruminococcus* or *R. bovis* to their hosts have been reported by the scientific community

(Appendix 017). These sequencing-based microbiome analyses from internal and external studies agree with our findings in Part 2.2, suggesting that feeding *R. bovis* ASCUSDY10 at its indented usage is safe.

6.1.3 *R. bovis* Supports Rumen Feed Digestion.

As described in Part 2.2 and Appendix 002, *In vitro* substrate utilization assays demonstrated that *R. bovis* ASCUSDY10 is a sugar fermenting organism, grows on a variety of simple and complex polysaccharides, including starch, maltose, fructose, and galactose (Part 2.2.1). *R. bovis* ASCUSDY10 is also capable of degrading starch (Part 2.2.1). Genomic analysis revealed that the *R. bovis* ASCUSDY10 genome encodes for amylosome, a complex enzymatic structure dedicated for starch adhesion and digestion, similar to those found in *R. bromii* (Mukhopadhya et al., 2018; Ze et al., 2015). Together, this data indicates that *R. bovis* contributes to ruminal polymer processing like other *Rumincoccus* species and supports the proposed role of *R. bovis* ASCUSDY10 as a source of viable microorganisms in the diet to support the digestion of carbohydrates, including ruminal starch.

6.1.4 *R. bovis* ASCUSDY10 is Shown Safe in Both *in silico* and *in vitro* Evaluations.

The genome of *R. bovis* ASCUSDY10 has been thoroughly interrogated for the presence of elements contributing to or part of microbial virulence including plasmids, antibiotic resistance and production, pathogenicity and virulence factors, defense metabolic products, stress response and dormancy. Four potential hemolysin family proteins were identified from this analysis (Part 2.2.7), and further *in vitro* assays confirmed that they are inactive and do not confer a hemolytic phenotype to *R. bovis* ASCUSDY10 under physiologically-relevant conditions. There are no other genetic elements with confirmed functions that could be linked to *R. bovis* ASCUSDY10 pathogenicity, virulence, protein toxins, or pro-inflammatory polysaccharides (Part 2.2.8). *R. bovis* ASCUSDY10 does not produce antimicrobial substances (Part 2.2.5 and Appendix 005). However, *R. bovis* ASCUSDY10 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin per EFSA interpretation (Part 2.2.4). It is important to mention that susceptibility to aminoglycosides (gentamicin, kanamycin, and streptomycin) and macrolides (erythromycin) decreases significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al. 2016). Therefore, this classification set forth by EFSA on aminoglycosides and macrolides should not be applied to *R. bovis* ASCUSDY10 due to its anaerobic nature (Part 2.2.5). *R. bovis* ASCUSDY10 is resistant to tetracycline (MIC less than 8 µg/ml), which is conferred by *tet(W)*, a ubiquitous gene in the bacterial population of the gastrointestinal microbiome of ruminants, humans, and other farm animals (Pal et al. 2016; Joyce et al. 2019; Sabino et al. 2019). Thus, based on the thorough screening of the *R. bovis* ASCUSDY10 genome using all applicable and relevant databases and the current state of the art, all potential concerns were identified and explained suggesting that *R. bovis* ASCUSDY10 is safe for humans and animals. In the unlikely event an opportunistic infection was caused by *R. bovis* ASCUSDY10, it could be treated with common antibiotics.

6.1.5 *Ruminococcus* Species Other Than *R. bovis* Have Been Safely Fed to Ruminants

Member of *Ruminococcus* have been supplemented in feed or administered directly to ruminants. Chiquette et al. (2007) found that supplementing *R. flavefaciens* in-feed to non-lactating dairy cows increased feed digestibility during the supplementation periods. No adverse health effects were observed.

In another study, beef steers receiving a high-grain diet were orally drenched with *R. bromii* and *Megasphaera elsdenii* (Klieve et al., 2012). The authors found no permanent establishment of *R. bromii* in rumen nor negative health impacts associated with microbial supplementation. Other studies conducted by Krause et al. (1999, 2001), Miyagi et al. (1995), and Kumar et al. (2021) have fed various strains of *R. flavefaciens* and *R. albus* to lambs, sheep, goats, and buffalos with no observed adverse health effects. These studies suggest that members of *Ruminococcus* are safe to be administered to ruminants, including dairy cows. It is also important to note that no pathogenicity or virulence has been reported for *Ruminococcus* species in literature to date, suggesting that *Ruminococcus* species exposure or consumption has no known, demonstrated, or predicted health risks (Appendix 017).

6.1.6 Supplementing *R. bovis* ASCUSDY10 in Feed Does Not Alter Rumen Microbiome Beyond Normal

The rumen microbiome is not static. In fact, the composition of the rumen microbiome is driven by multiple factors including the host genetics, diet and geographic location (Henderson et al., 2015). From both published literature and Native Microbials' microbiome surveys, *Actinobacteria*, *Bacteroidetes*, *Fibrobacteres*, *Firmicutes*, *Proteobacteria*, *Spriochaetes*, and *Tenericutes* are the dominant bacterial phyla in rumen (Appendix 018). Feeding trials sponsored by Native Microbials showed that supplementing *R. bovis* ASCUSDY10 in feed did not alter the abundance of the core bacterial phyla beyond their normal ranges (Appendix 018). Therefore, the dietary addition of *R. bovis* ASCUSDY10 will not cause a safety concern based on changes in the rumen microbiome.

6.2 Target Animal Feeding Studies Including *R. bovis* ASCUSDY10

Six independent studies sponsored by Native Microbials, involving 589 lactating dairy cows, are presented here to corroborate target animal safety. Rumen native microorganisms, including *R. bovis* ASCUSDY10 were supplemented in-feed or top dressed to lactating dairy cows daily across a range of time (16-39 weeks of treatment) periods as summarized in Table 6.1 and described below in Part 6.2.1 to 6.2.6:

Table 6.1: Summary of the Target Animal Feeding Studies Including *R. bovis* ASCUSDY10					
Study	**DFM Administration Method**	**Feeding Period**	**Treatment Groups**	**Number of animals**	***R. bovis* ASCUSDY10 Dosage (CFU/d/cow)**
(b) (4) (Appendix 019A	Mixed in-feed using a mixing wagon	39 weeks	Control	30	0
			MFS1	30	0
			MFS2	30	1.E+08
(b) (4) (Appendix 019B)	Top-dressed	16 weeks	Control	30	0
			G2	30	1.E+08
			G2P	30	1.E+08
(b) (4) (Appendix 019C)	Top-dressed	20 weeks	Control	24	0
			MFS1	24	0
			MFS2	24	1.E+08
(b) (4) (Appendix 019D)	Top-dressed	20 weeks	Control	39	0
			G1	39	0
			G2	39	1E+08
(b) (4) (Appendix 019E)	Top-dressed	20 weeks	Control	30	0
			GF	30	1.E+08
(b) (4) (Appendix 019F)	Mixed in-feed using a mixing wagon	20 weeks	Control	74 (5 pens)	0
			MFA	76 (5 pens)	1.E+08

6.2.1 Study (b) (4) (Published Study Report – Appendix 019A)

In the first study, 90 multiparous (2 or 3 lactation cycles) lactating Holstein cows (20-40 days in milk) were sourced from a large commercial dairy farm and housed in a single pen equipped with (b) (4) gates at (b) (6), (b) (4). The cows were divided into 3 groups, 30 of which served as control (Control: no microbes), 30 received a DFM consisting of 2 microbes (MFS1: no *R. bovis* ASCUSDY10), and the remaining 30 cows received a DFM consisting of 4 microbes (MFS including *bovis* ASCUSDY10 at 1E8 CFU/d/cow). Both DFMs were in powder form and were homogeneously mixed into the feed prior to administration via mixing wagon. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered in-feed to lactating dairy cows daily for 39 weeks. The health condition of the animals was closely monitored and recorded. Rumen samples were collected from all animals on day 270. Additional observations include milk yield, milk components, dry matter intake, body weight, body condition scores, and pregnancy (Valldecabres et al., 2022; Appendix 019A).

No significant differences in clinical mastitis occurrence were observed among treatment groups. The estimated risk ratios also indicated that no risk of clinical mastitis was associated with any of the treatment

groups. No adverse effects were reported for any of the variables measured over the duration of the study. Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.2 Study (b) (4) (Published Study Report – Appendix 019B)

The second study involving 90 primiparous and multiparous lactating Holstein cows 92±23 days in milk was conducted at (b) (6), (b) (4). The animals were divided into 3 groups, 30 of which served as control (Control: no microbes), 30 received a DFM consists of 4 microbes at minimum label claim daily (G2: including *R. bovis* ASCUSDY10 at 1E8 CFU/d/cow), the remaining 30 cows received the same DFM but at the commercial usage level daily (G2P: including *R. bovis* ASCUSDY10 at 1E8 CFU/d/cow as well). Both DFMs were in powder form and were top-dressed onto the feed prior to the morning feeding. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to lactating dairy cows daily for 112 days. The health condition of the animals was closely monitored and recorded. Additional observations include milk yield, milk components, dry matter intake, body weight, body condition scores, and total tract digestibility. Nine cows from each group were selected and followed for rumen sample collection on day 0 (prior to the administration of microbes) and day 112 (Goldsmith et al., 2023; Appendix 019B).

During the study, one cow in G2P had a teat injury and developed mastitis due to *Trueperello* (*Arcanobacterium*) *pyogenes* and hindlimb lameness later (Appendix 019B). No other cases of clinical mastitis were reported. No adverse effects were reported for any of the other variables measured over the duration of the study. Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.3 Study (b) (4) (Published Study Report – Appendix 019C)

The third study was conducted at (b) (6), (b) (4) using 72 (1 additional cow as enrolled as backup) primiparous and multiparous lactating Holstein dairy cows. The animals were divided into 3 groups, 24 of which served as control (Control: received no microbes), 24 of which received a DFM consisting of 2 microbes daily (MFS1: no *R. bovis* ASCUSDY10), and the remaining 24 cows received a DFM consisting of 4 microbes daily (MFS2: including *R. bovis* ASCUSDY10 at 1E8 CFU/d/cow). Both DFMs were in powder form and were top-dressed onto the feed prior to the morning feeding. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to cows for 20 weeks. The health condition of the animals was closely monitored and recorded. Rumen samples were collected from the same 15 cows of each group before the microbes were administered, during week 6, week 11, and week 20. Additional observations include milk yield, milk components, dry matter intake, body weight, and body condition scores (Dickerson et al., 2022; Appendix 019C).

No adverse effects were reported for any of the variables measured over the duration of the study between control and treatment group (Appendix 019C). Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.4 Study (b) (4) (Unpublished study – Appendix 019D)

The fourth study was conducted at (b) (4) using 117 primiparous and multiparous lactating Holstein dairy cows. The animals were divided into 3 groups, 39 of which served as control (CON: received

no microbes), 39 of which received a DFM consisting of 2 microbes daily (G1: no *R. bovis* ASCUSDY10), and the remaining 39 cows received a DFM consisting of 4 microbes daily (G2: including *R. bovis* ASCUSDY10 at 2E8 CFU/d/cow). Both DFMs were in powder form and were top-dressed onto the feed prior to the morning feeding. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to cows for 20 weeks. The health condition of the animals was closely monitored and recorded. Rumen samples were collected from all cows before the microbes were administered, during week 10 and week 20. Additional observations include milk yield, milk components, dry matter intake, body weight, and body condition scores (D).

No adverse effects were reported for any of the variables measured over the duration of the study versus the control (Appendix 019D). Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.5 Study (b) (4) (Unpublished study – Appendix 019E)

The fifth study was conducted at (b) (4) using 60 Holstein dairy cows. The animals were divided into 2 groups, 30 of which were served as control (CON: no microbes), and the remaining 30 cows received a DFM consisting of 4 microbes daily (GF: including *R. bovis* ASCUSDY10 at 1E8 CFU/d/cow). The DFM was in powder form and was top-dressed onto the feed prior to the morning feeding. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to cows daily during the dry period (21±3 days precalving) and until 120±3 days in milk. The health condition of the animals (including the occurrence of mastitis) was closely monitored, and blood samples were collected for the evaluation of energy and nitrogen metabolism, liver function, inflammation and oxidative stress. Additional observations include milk yield, milk components, dry matter intake, body weight, body condition scores, and apparent digestibility. Rumen samples were collected from the same 12 cows of each treatment group during precalving period (-14±3 day and -7±3 day) and lactating period (7±3 days in milk, 14±3 days in milk, 21±3 days in milk, 70 days in milk, and 100 days in milk)

No adverse effects were reported for any of the variables measured over the duration of the study (Appendix 019E). Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.6 Study (b) (4) (Presented at conference - Appendix 019F)

The sixth study was conducted at (b) (4) using 150 primiparous and multiparous Holstein cows. The animals were divided into two groups, 74 of which (5 pens with 15-16 cows/pen) were served as control (CON: received no microbes), 76 of which (5 pens with 15-16 cows/pen) received a DFM consisting of 4 microbes daily (MFA: including *R. bovis* ASCUSDY10 at 1E8 CFU/d/cow). The DFM was in powder form and was homogeneously mixed into the feed prior to feeding. Native rumen microbes, *R. bovis* ASCUSDY10 was administered to lactating dairy cows for 20 weeks. The health condition of the animals was closely monitored and recorded. Additional observations include milk yield, milk components, dry matter intake, body weight, and body condition scores. Rumen samples were collected from the same 2 cows per each pen before the microbes were administered, on day 70 and day 100 of the treatment period (Ferro et al., 2022; Appendix 019F).

No adverse effects were reported for any of the variables measured over the duration of the study (Appendix 019F). Overall, the findings of the study corroborate the safety of *R. bovis* ASCUSDY10 for dairy cows.

6.2.7 Feeding Studies Summary

Overall, the study findings provide corroborative evidence that *R. bovis* ASCUSDY10 is well-tolerated and does not cause adverse effects when fed to dairy cows. Together, these studies verify the assessment of safety.

6.3 GRAS Panel Evaluation

Native Microbials has concluded that fat-encapsulated *R. bovis* ASCUSDY10 is GRAS for dairy cattle as previously described. This conclusion is based on data available in the public domain as well as that demonstrated and presented by Native Microbials. In addition, this conclusion is affirmed by a consensus of experts (GRAS Panel) convened to evaluate the safety of four commensal dairy organisms toward the target animal (Dairy Cattle) to the State of Texas, which included *R. bovis* ASCUSDY10. The panelists were qualified by scientific training, experience and expertise. The GRAS panel consisted of the three scientific experts: Bradley J Johnson, PhD, the current Gordon W. Davis Regent's Chair in Meat Science and Muscle Biology and Professor in the Davis College of Agricultural Sciences and Natural Resources' Department of Animal and Food Sciences at Texas Tech University; T.G Nagaraja, BVSc, PhD, University Distinguished Professor and the Roy Walter Upham Endowed Professor in the Department of Diagnostic Medicine and Pathobiology in the College of Veterinary Medicine at Kansas State University; and Jhones O Sarturi, Ph.D., who received his DVM from the University for the Development of Pantanal – Brazil (UNIDERP), M.S in Agronomy from the University of Sao Paulo Brazil (USP/ESALQ), and a Ph.D in Animal Science from the University of Nebraska Lincoln (UNL) and worked as a Post-Doctoral Research Associate at Texas A&M, currently a tenured Associate Professor at Texas Tech University, Department of Animal and Food Sciences.

The panel convened to evaluate all data presented by Native Microbials, including data presented in this GRAS Dossier, to conduct a risk assessment for the microorganisms (which included *R. bovis* ASCUSDY10) for use as direct-fed microbial for cattle. Their detailed conclusions are found in Appendix 20. In part, the panel concluded that, 1) The strains (including *R. bovis* ASCUSDY10) belong to species that are members of the normal microbial community of dairy cattle, 2) The strains (including *R. bovis* ASCUSDY10) are closely related to the other strains of the species prevalent in the rumen of cattle, 3) The strains (including *R. bovis* ASCUSDY10) do not contain virulence genes that code for toxins or other independent virulence factors that may contribute to pathogenicity, 4) The strains (including *R. bovis* ASCUSDY10) are not likely to contribute to the antimicrobial resistance (AMR) of bacteria or fungi in the gastrointestinal tract of cattle or the environment, 5) Safety of the strains (including *R. bovis* ASCUSDY10) is supported by studies conducted at universities that have evaluated their impact on milk production in dairy cows, and 6) Safety of the strains (including *R. bovis* ASCUSDY10) is supported by assessments reported by the company that indicated no negative influence on the ruminal microbiome.

6.4 Overall Summary of Safety

R. bovis ASCUSDY10 belongs to the species *R. bovis* and is a ubiquitous and prevalent member of the rumen microbiome. *R. bovis* is naturally present in the rumen and is prevalent in the gut of a variety of ruminant species across the globe. It has been detected in published microbiome sequencing data (Appendix 018) and in-house microbiome sequencing surveys, despite the fact that the microorganism was only formally named recently (Gaffney et al., 2021). The whole genome sequence analysis of *R. bovis* ASCUSDY10 indicates the absence of active genes involved in toxin production or other virulence factors known to be associated with pathogenicity, which was corroborated with *in vitro* testing and multiple feed studies where *R. bovis* ASCUSDY10 was fed to cattle with no adverse effects. Furthermore, no transfer of viable *R. bovis* ASCUSDY10 from the rumen to milk or other edible tissues is anticipated under the conditions of intended use as a direct fed microbial in the feed of dairy cattle, nor would such transfer be pathogenic to humans. Taken together, these data indicate that *R. bovis* ASCUSDY10 (the notified substance) should not be associated with any safety concerns for dairy cattle or any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of dairy cattle.

In this safety assessment we identified, discussed, and placed into context data and information that are, or may appear to be inconsistent with the GRAS status (21 CFR 570.250(c)(1)). Based on the preponderance of evidence, Native Microbials' conclusion of safety is scientifically justified.

PART 7 – LIST OF SUPPORTING DATA AND REFERENCES

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Dr. Mallory Embree
Ascus BioSciences
6450 Lusk Blvd.
Suite E109/209
San Diego, CA 92121
United States

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ***Ruminococcus spp.*** ***(Ascusb_5)***	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **NRRL B-67764**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

☐ [1]a scientific description

☒ [1]a proposed taxonomic designation

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on **April 11, 2019** (date of the original deposit)[2]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above, was received by this International Depositary Authority on _____ (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Agricultural Research Culture Collection (NRRL) International Depositary Authority Address: 1815 N. University Street Peoria, Illinois 61604 U.S.A.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): (b) (6) Date: April 26, 2019 **Travis W. Adkins**

[1] Mark with a cross the applicable box.

[2] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

Dr. Mallory Embree
Ascus BioSciences
6450 Lusk Blvd.
Suite E109/209
San Diego, CA 92121
United States

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Dr. Mallory Embree Ascus BioSciences 6450 Lusk Blvd. Suite E109/209 San Diego, CA 92121 United States	Depositor's taxonomic designation and accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY ***Ruminococcus spp.* (Ascusb_5)** **NRRL B-67764** Date of: **April 11, 2019** ☒ Original Deposit ☐ New Deposit ☐ Repropagation of Original Deposit

III. (a) VIABILITY STATEMENT

Deposit was found:
☒ [2]Viable ☐ [2]Nonviable on _____ (Date)
International Depositary Authority[4]'s preparation was found viable on **April 11, 2019** (Date)[3]

III. (b) DEPOSITOR'S EQUIVALENCY DECLARATION

Depositor determined the International Depositary Authority's preparation was
☐ [2]Equivalent
☐ [2]Not equivalent to deposit on NA (Date)

Signature of Depositor

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST WAS PERFORMED (Depositors/Depositary)[4]

original stocks were used for the deposit after we verified growth from those stocks, so there will be no need to send back verification stocks for your viability.

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Agricultural Research Culture Collection (NRRL) International Depositary Authority Address: 1815 N. University Street Peoria, Illinois 61604 U.S.A.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): (b) (6) **Date: April 26, 2019** **Travis W. Adkins**

[1] Indicate the date of the original deposit or when a new deposit has been made.
[2] Mark with a cross the applicable box.
[3] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
[4] Fill in if the information has been requested.

Appendix 002: Supplementary Methods and Results for *R. bovis* ASCUSDY10 In Vitro Biochemical Assays

Objectives:

The objective of this work was to assess the carbohydrate fermentation capabilities of *R. bovis* ASCUSDY10 through in vitro assays.

Methods:

Carbohydrate fermentation of *R. bovis* ASCUSDY10 was qualitatively measured using the (b) (4) carbon panel ((b) (6), (b) (4)). Results can be found in Table 1. *R. bovis* ASCUSDY10 cells were grown to late exponential phase and recovered by centrifugation at 3,000 x g for 10 minutes. Cells were resuspended and (b) (4) (wt/vol) bromocresol purple added as a pH indicator for acidification of carbohydrates (Avgustin et al. 1997).

Metabolite production of *R. bovis* ASCUSDY10 fermentation run 1801.2038 was measured at 5 ,9, 17.1, 25, 32 hours using an (b) (4) series with RI detector operated at 35°C. The column used was a (b) (4) #1250140 with (b) (4) guard #1250129 operated at 60°C. The mobile phase was (b) (4) N Sulfuric Acid ((b) (4) mL concentrated sulfuric to (b) (4) at a flow rate of (b) (4) mL/min. Pure standards were used at varying concentrations to generate a standard curve.

Results:

R. bovis ASCUSDY10 was assessed for fermentation of 50 carbon sources. Carbon source fermentation data is shown below in table 1.

Table 1. Carbon Source Fermentation by *R. bovis* ASCUSDY10

Carbon Source	Growth	Carbon Source	Growth
No Carbon Control	No Growth	Inositol	No Growth
Glycerol	No Growth	D-Mannitol	No Growth
Erythritol	No Growth	D-Sorbitol	No Growth
D-Arabinose	No Growth	Methyl-aD-Mannopyranoside	No Growth
L-Arabinose	No Growth	Methyl-aD-Glucopyranoside	No Growth
D-Ribose	No Growth	N-AcetylGlucosamine	No Growth
D-Xylose	No Growth	Amygdalin	No Growth
L-Xylose	No Growth	Arbutin	No Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth
Methyl-BD-xylopyranoside	No Growth	Salicin	No Growth

D-Galactose	Growth	D-Cellobiose	No Growth
D-Glucose	Growth	D-Maltose	Growth
D-Fructose	Growth	D-Lactose	No Growth
D-Mannose	No Growth	D-Melibiose	No Growth
L-Sorbose	No Growth	D-Saccharose	No Growth
L-Rhamnose	No Growth	D-Trehalose	No Growth
Dulcitol	No Growth	Inulin	No Growth
D-Melezitose	No Growth	D-Tagatose	No Growth
D-Raffinose	No Growth	D-Fucose	No Growth
Starch	Growth	L-Fucose	No Growth
Glycogen	Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	No Growth	Potassium Gluconate	No Growth
D-Turanose	No Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth		

Table 2. Metabolite Production by *R. bovis* ASCUSDY10 on Complex Media with Maltose

	Maltose	Pyruvic Acid	Succinic Acid	Lactic Acid	Glycerol	Acetic Acid	Propionic Acid	Butyric Acid	1-Butanol	Ethanol
Fermentation Time (hrs)	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L
5.0										
9.0										
17.1										
25.0										
32.0										

(b) (4)

Conclusions:

In vitro assays demonstrate that *R. bovis* ASCUSDY10 grows on a variety of mono and polysaccharides including galactose, glucose, fructose, starch, glycogen, and maltose. Additionally, the species also grows on esculin/ferric citrate. When grown on maltose *R. bovis* ASCUSDY10 produces acetate and ethanol as major fermentation products.

Signed: (b) (6)_____ Date: _____

References

Avgustin, G., R. J. Wallace, and H. J. Flint. 1997. "Phenotypic Diversity among Ruminal Isolates of Prevotella Ruminicola: Proposal of Prevotella Brevis Sp. Nov., Prevotella Bryantii Sp. Nov., and Prevotella Albensis Sp. Nov. and Redefinition of Prevotella Ruminicola." *International Journal of Systematic Bacteriology*. https://doi.org/10.1099/00207713-47-2-284.

Appendix 003A: Supplemeutary Determiue the Identity of *Ruminococcus bovis* ASCUSDY10 Using Genomic Methods

For 16S sequence analysis, the 16S gene was amplified from *R. bovis* ASCUSDY10 the 27F/534R primers and sequenced using an (b) (4) (Stackebrandt and Goodfellow 1991; Muyzer, de Waal, and Uitterlinden 1993; LANE and J 1991). The resulting sequence was quality trimmed and compared to NCBI databases (excluding "uncultured" and environmental samples) to establish the identity of the strain. The NCBI databases were queried on November 29, 2023.

Genomic DNA was isolated from a pure culture of *R. bovis* ASCUSDY10 by a modified Sambrook phenol-chloroform extraction/purification protocol (Jain et al. 2018). Short read sequencing libraries were prepared using the (b) (6), (b) (4)) by manufacturer's recommended protocol and the resulting libraries were sequenced (1x300bp) on an (b) (4). In parallel, long read libraries were prepared from the same extracted DNA using the (b) (4), (b) (6) using a modified version of the protocol outlined by (Jain et al. 2018) and 1D sequenced on the (b) (4). Full details of the genome assembly can be found in Appendix 003C. (b) (4) and (b) (4) were used to generate the alignments for whole genome average nucleotide identity (ANI) (Lee et al., 2016; Kurtz et al., 2004).

Signed: (b) (6) Date: _____

References

Jain, Miten, Sergey Koren, Karen H. Miga, Josh Quick, Arthur C. Rand, Thomas A. Sasani, John R. Tyson, et al. 2018. "Nanopore Sequencing and Assembly of a Human Genome with Ultra-Long Reads." *Nature Biotechnology* 36 (4): 338–45.

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LANE, and D. J. 1991. "16S/23S rRNA Sequencing." *Nucleic Acid Techniques in Bacterial Systematics*. https://ci.nii.ac.jp/naid/10005795102.

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Yarza, Pablo, Pelin Yilmaz, Elmar Pruesse, Frank Oliver Glöckner, Wolfgang Ludwig, Karl-Heinz Schleifer, William B. Whitman, Jean Euzéby, Rudolf Amann, and Ramon Rosselló-Móra. 2014. "Uniting the Classification of Cultured and Uncultured Bacteria and Archaea Using 16S rRNA Gene Sequences." *Nature Reviews. Microbiology* 12 (9): 635–45.

Yoon, Seok-Hwan, Sung-Min Ha, Jeongmin Lim, Soonjae Kwon, and Jongsik Chun. 2017. "A Large-Scale Evaluation of Algorithms to Calculate Average Nucleotide Identity." *Antonie van Leeuwenhoek* 110

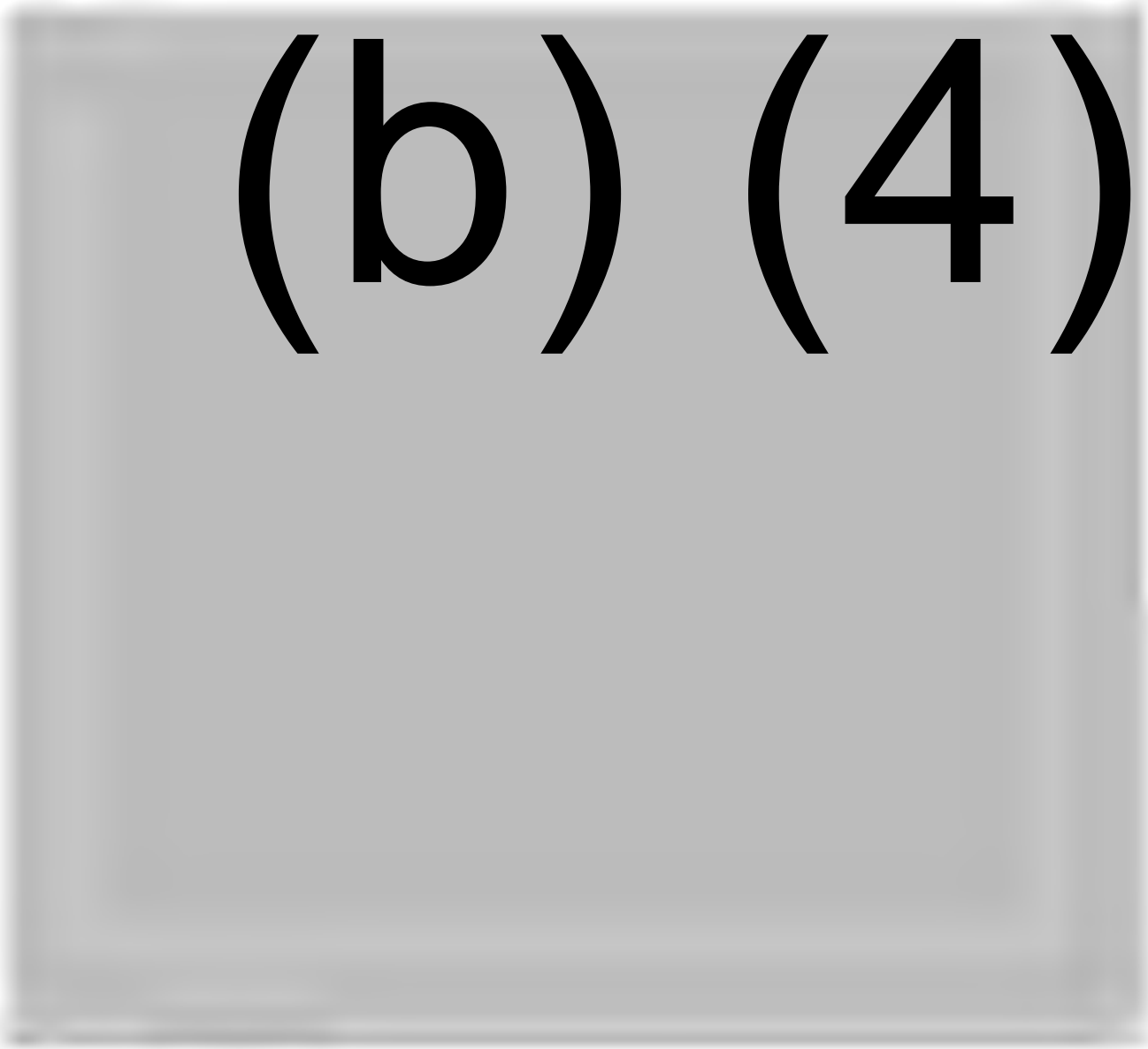
Appendix 003B: Supplementary Whole Genome Analysis Methods and Read QualityMetrics for *R. bovis* ASCUSDY10

The *R. bovis* ASCUSDY10 genomic DNA was extracted and sequenced as described in the main text of the dossier. This appendix contains details about the assembly methods used, the protocol for (b) (4) library preparation, (b) (4) quality metrics for the (b) (4) and (b) (4) reads respectively, metrics generated by (b) (4) for the completed assembly, and a visualization of the assembly graph generated by (b) (4).

Assembly Pipeline in Detail

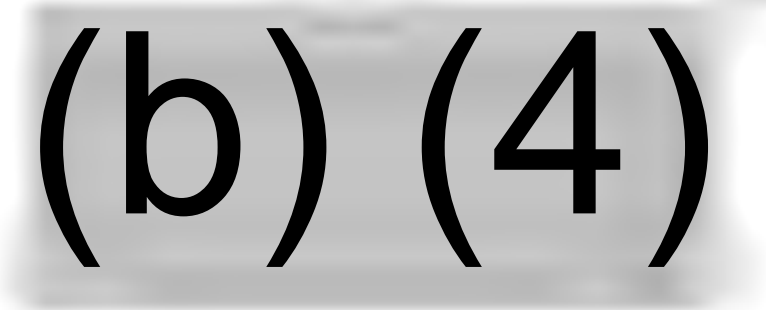
1. (b) (4)
2.
3.
4.
5.
6.
7.
8.

(b) (4) Protocol as Provided by the Manufacturer

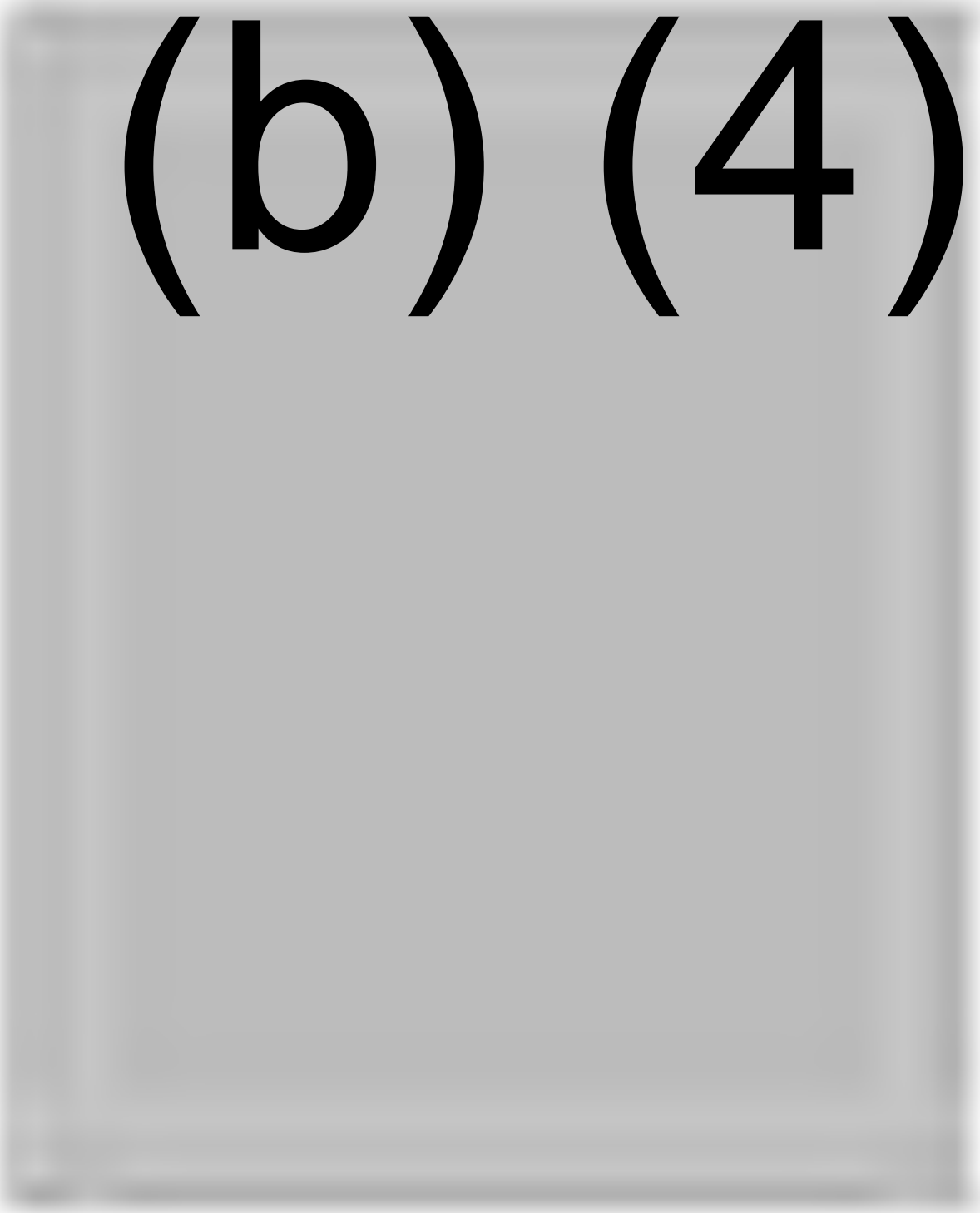


Full Protocol: (b) (4)

Quality Metrics of (b) (4) Reads as Generated by (b) (4)



Read distribution as related to quality score



Metrics for *R. bovis* ASCUSDY10 (b) (4) **reads as generated by** (b) (4)

General Summary

(b) (4)

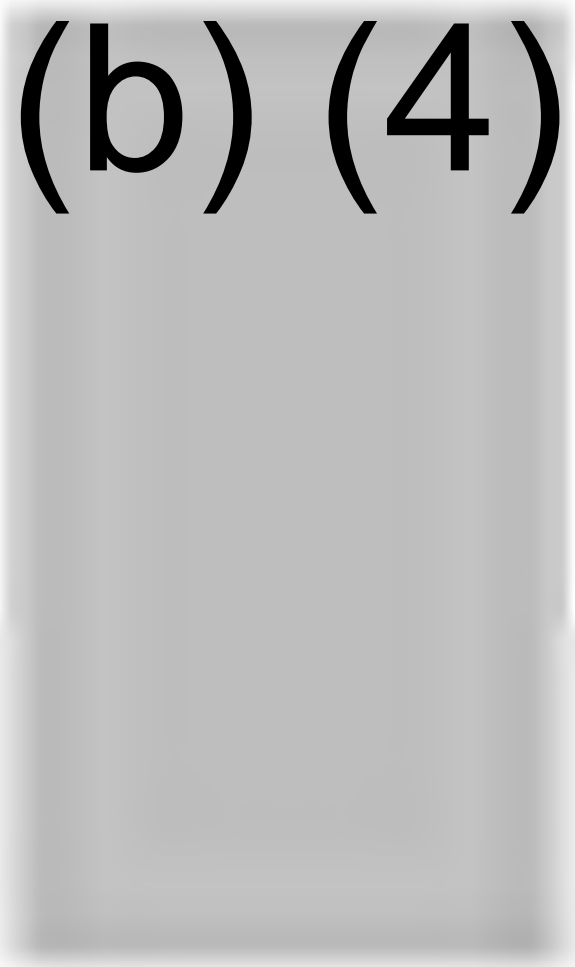
Number, Percentage, and Megabases of Reads Above Quality Cutoffs

(b) (4)

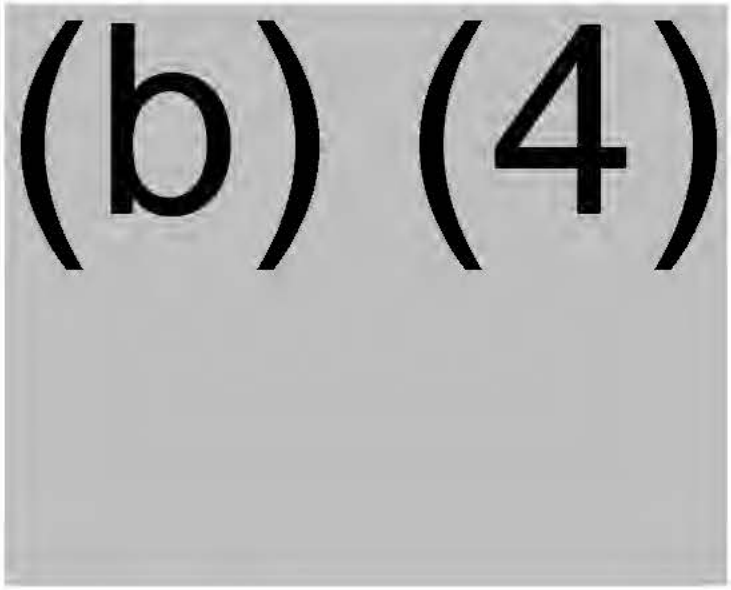
Longest Reads in Base Pairs (bp)

(b) (4)

Assembly Statistics as reported by (b) (4)



Assembly Graph as Visualized by Bandage.



Signed (b) (6) _____ Date: _____

References

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M. and Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics*, *34*(15), pp.2666-2669.

Gurevich, A., Saveliev, V., Vyahhi, N. and Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), pp.1072-1075.

Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H. and Phillippy, A.M., 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome research*, *27*(5), pp.722-736.

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Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C.A., Zeng, Q., Wortman, J., Young, S.K. and Earl, A.M., 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PloS one*, *9*(11), p.e112963.

Wick, R.R., Schultz, M.B., Zobel, J. and Holt, K.E., 2015. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics*, *31*(20), pp.3350-3352.

Appendix 003C Ruminococcus bovis NCBI Accession

Download Assembly

ASM560113v1

Organism name: Ruminococcus sp. JE7A12 (firmicutes)
Infraspecific name: Strain: JE7A12
BioSample: SAMN11351985
BioProject: PRJNA531197
Submitter: Ascus Biosciences
Date: 2019/05/22
Assembly type: na
Assembly level: Complete Genome
Genome representation: full
GenBank assembly accession: GCA_005601135.1 (**latest**)
RefSeq assembly accession: GCF_005601135.1 (**latest**)
RefSeq assembly and GenBank assembly identical: yes
Assembly method: Canu v. 1.8
Expected final version: yes
Genome coverage: 50.0x
Sequencing technology: (b) (4)

IDs: 3018681 [UID] 10322038 [GenBank] 10710288 [RefSeq]

See Genome Information for **Ruminococcus sp. JE7A12**

History (Show revision history)

Comment

The annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Information about PGAP can be found here: https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

Genome-Annotation-Data

See more

Global statistics

Total sequence length	2,440,231
Total ungapped length	2,440,231
Total number of chromosomes and plasmids	1

Assembly Definition | Assembly Statistics

Global assembly definition

Download the full sequence report

Assembly Unit: Primary Assembly (GCF_005601165.1)

Molecule name	GenBank sequence		RefSeq sequence
Chromosome	CP039381.1	=	NZ_CP039381.1

Characterization of Native Microbials *Ruminococcus bovis* ASCUSDY10 (Dairy-10) Production Strain: Antibiotic Susceptibility Profile

Approvers:

(b) (6) _____ 12/22/2020

Research Scientist Date

(b) (6) _____ 12/22/2020

Quality Date

(b) (6) _____ 12/22/2020

Kevin Korth
Regulatory Date

Table of Contents

Title: Characterization of Native Microbials *Ruminococcus bovis* ASCUSDY10 (Dairy-10) Production Strain: Antibiotic Susceptibility Profile

1 OBJECTIVE

To determine the Susceptibility Profile of *Ruminococcus bovis* (Dairy-10) production strain to European Food Safety Authority recommended antimicrobials.

2 STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01 and M11 to the extent to which it is applicable as detailed in the protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype minimal inhibitory concentration (MIC) values. Procedures for the susceptibility were designed to follow those in European Food Safety Authority (EFSA) *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA Panel on Additives and Products or Substances used in Animal Feed [FEEDAP] Rychen et al., 2018) as applicable and as detailed in the protocol.

3 STUDY SITE

Antimicrobial susceptibility testing was performed at Native Microbials Inc.

4 MATERIALS AND METHODS

4.1 Isolate

A production strain of *Ruminococcus bovis* ASCUSDY10 (ASCUSDY10) was procured from the 20Sep20 Commercial Working Cell Bank. The culture was streaked onto both Brucella agar and Mueller Hinton agar to verify that the organism is viable, pure, and morphologically typical of the purported species and to verify growth on the selected media.

4.2 Susceptibility Profile

4.2.1 Procedure

The procedures listed in the protocol “Agar-Dilution Susceptibility Testing of Anaerobes” (Appendix A) were written to comply with CLSI document VET01 entitled *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals* and CLSI Document M11 entitled *Methods of Antimicrobial Susceptibility Testing of Anaerobic Bacteria*.

4.3 Media

MIC agar plates for use in an agar dilution method were prepared by Native Microbials with antimicrobials and doubling dilution concentrations. The media for MIC testing was Brucella Broth. Stock solution concentrations and media recipes are captured in Appendix B.

4.4 Incubation and Interpretation of Susceptibility Tests

MIC agar plates were incubated and interpreted according to Native Microbials internal protocol "Agar-Dilution Susceptibility Testing of Anaerobes" (Appendix A).

Sensitivities were compared to applicable values (Table 1) from EUCAST clinical breakpoints for gram positive anaerobes ("Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 10.0", 2020), CLSI breakpoints for anaerobic organisms (Clinical and Laboratory Standards Institute [CLSI], 2020), and EFSA breakpoints for gram-positive bacteria (Rychen et al., 2018).

4.5 Quality Control

Reference Strain *Escherichia coli* (ATCC 25922) was tested on each agar dilution plate to ensure proper quality control (QC) of the MIC tests. Available CLSI (CLSI, 2020) and EUCAST ("Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion as Recommended by EUCAST, Version 10.0"; EUCAST, 2020) acceptable QC ranges for each antimicrobial were referenced (Table 3).

With each test, all growth was verified to be of one morphology and of the correct colony morphological features as considered typical of the strain.

Table 1. EFSA Gram Positive Breakpoints, EUCAST Gram-Positive Anaerobic Breakpoints and CLSI Anaerobes Breakpoints.

	EFSA Gram-Positive		EUCAST Gram-Positive Anaerobes		CLSI Anaerobes		
Antibiotic	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**I**	**R >**
Ampicillin	1	1	4	8	0.5	1	2
Vancomycin	4	4	2	2			
Gentamicin	4	4	-	-			
Kanamycin	16	16					
Streptomycin	8	8					
Erythromycin	1	1	-	-			
Clindamycin	4	4	4	4	2	4	8
Tetracycline	2	2			4	8	16
Chloramphenicol	4	4	8	8	8	16	32

5 DISPOSITIONS

All agar dilution plates were discarded after their expiration. The isolate and all subcultures were discarded after autoclaving. No retention cultures were created or maintained from this study.

6 RESULTS

MIC results of the *Ruminococcus bovis* ASCUSDY10 (Dairy-10) isolate and breakpoints interpretations are presented in Table 2. Photographs of agar dilution plates are shown in Appendix C. The isolate would be considered wild-type or susceptible according to all three criteria (EFSA, EUCAST, and CLSI) to Ampicillin, Clindamycin and Chloramphenicol. The isolate would be considered susceptible to Vancomycin according to EFSA and EUCAST breakpoints. According to CLSI, the isolate would be intermediately sensitive to Tetracycline, although would be considered resistant to EFSA. The isolate would be considered non-wildtype or non-susceptible, against Gentamicin, Kanamycin, Streptomycin and Erythromycin to EFSA.

However, one must consider that some classifications set forth by EFSA are for general Gram-Positive organisms and are not applicable to *Ruminococcus bovis* due to its anaerobic nature. EUCAST provides a breakpoint of "-" for Gentamicin and Erythromycin (Table 1) indicating that the species is a poor target for therapy with these antibiotics. CLSI refrains from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. It is well documented that aminoglycosides are hindered by anaerobic growth. Active electron transport is required for aminoglycoside uptake into cells, so the class inherently lacks activity against anaerobic bacteria (Kislak, 1973; Martin, Gardner, and Washington, 1972; Ramirez and Tolmasky, 2010). Susceptibility to aminoglycosides and macrolides decreases significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al., 2016).

Table 2. Minimal Inhibitory Concentrations for *Ruminococcus bovis* and Sensitivity Interpretation

			Interpretation		
Antibiotic	Range Tested (ug/mL)	Ruminococcus bovis	EFSA	EUCAST	CLSI
Ampicillin	0.5 - 128	< 0.5	S	S	S
Vancomycin	0.125 - 32	2	S	S	
Gentamicin	0.5 - 32	> 32	R	-	
Kanamycin	0.5 - 64	> 64	R		
Streptomycin	0.5 - 64	> 64	R		
Erythromycin	0.5 - 16	8	R	-	
Clindamycin	0.03 - 32	0.06	S	S	S
Tetracycline	0.0625 - 64	8	R		I
Chloramphenicol	0.5 - 64	2	S	S	S

MIC results of the QC strain ATCC 25922 *Escherichia coli* are presented in Table 3. ATCC 25922 performed within the expected range for Ampicillin, Tetracycline and Chloramphenicol. When compared to QC ranges for the aminoglycosides, Gentamicin, Kanamycin and Streptomycin, it appears to be out of specification. However, ATCC 25922 is a facultative anaerobe and in this testing, was grown in an anaerobic environment. The QC range provided by CLSI and EUCAST are for aerobic growth of ATCC 25922. For the reasoning provided above, these results are to be expected and are not indicative of a failure in the agar dilution plates.

The MIC results for the quality control organism is within the expected values, knowing that aminoglycosides (gentamicin, kanamycin and streptomycin) and macrolides (erythromycin) have reduced efficacy in anaerobic conditions.

Table 3. Minimal Inhibitory Concentrations for QC Strain ATCC 25922

Antibiotic	ATCC 25922	CLSI QC Ranges (µg/mL)	EUCAST QC Range (µg/mL)
Ampicillin	(b) (4)	2-8	2-8
Vancomycin			
Gentamycin		0.25 - 1	0.25 - 1
Kanamycin		1 -4	
Streptomycin			
Erythromycin			
Clindamycin			
Tetracycline		0.5 - 2	
Chloramphenicol		2-8	2-8

7 REFERENCES

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Appendix A. Agar-Dilution Susceptibility Testing of Anaerobes

1 General Considerations

1.1 The procedures described herein are designed to follow those described in Clinical & Laboratory Standards Institute (CLSI) document M11: Anaerobic Bacteria Antimicrobial Susceptibility.

1.2 Agar-dilution method is considered the standard method of antimicrobial susceptibility testing of anaerobic bacteria by CLSI.

1.2.1 Anaerobic organisms commonly require complex nutritional formulations for growth. Organisms to be assayed using this method need to be tested for growth on Mueller-Hinton Agar or Supplemented Brucella Agar. Supplements should not be used unless necessary for the growth of the organism. The use of other media is not recommended due to potential interference between antibiotics and media components (e.g. p-aminobenzoic acid, thymidine, glycine, divalent cations).

1.3 Unless otherwise noted, perform all work in an anaerobic chamber using degassed supplies.

1.4 Organisms will be grown on pre-reduced agar as appropriate for the particular strain (Reinforced Clostridial Agar, Tryptic Soy Agar, etc.). Organisms that are more aerotolerant may be grown on non-reduced agar.

1.4.1 To reduce media for testing, place agar plates or liquid media into an anaerobic chamber overnight. A reducing agent may be added to liquid media to expedite oxygen removal. An anaerobic indicating dye may be used in both agar or liquid media to provide a visual cue for reduced media.

2 Media Preparation

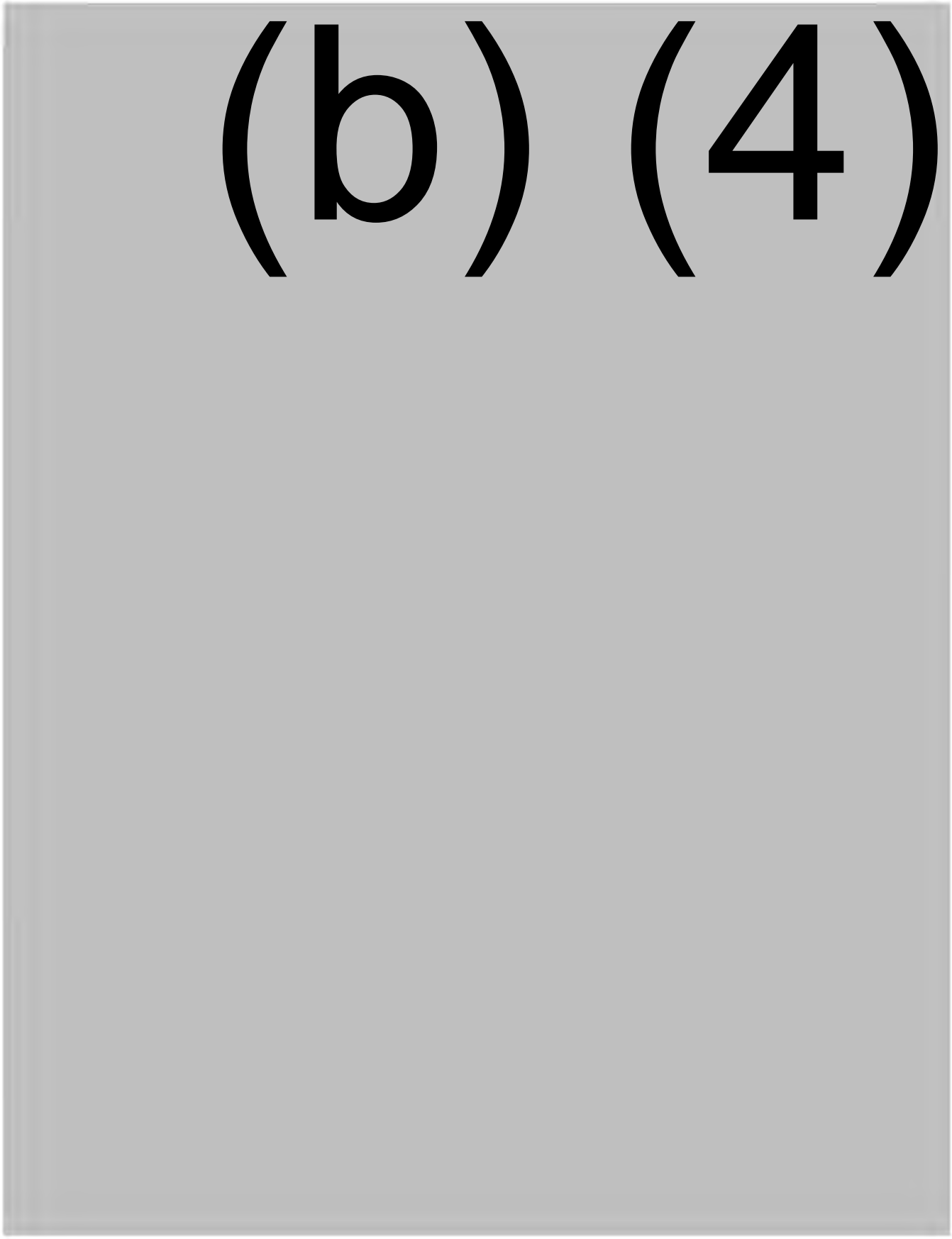
(b) (4)

(b) (4)

Table 1. Preparation of Dilutions of antimicrobial agents for use in agar dilution susceptibility tests.

Antimicrobial concentration (µg/mL) in stock	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (µg/mL)	Final Concentration in Agar (µg/mL)
(b) (4)				–
				–
				–
				–
				512
				256
				128
				64
				32
				16
				8
				4
				2
				1
				0.5
				0.25
				0.125
				0.06
				0.03
				0.015
				0.008
				0.004

(b) (4)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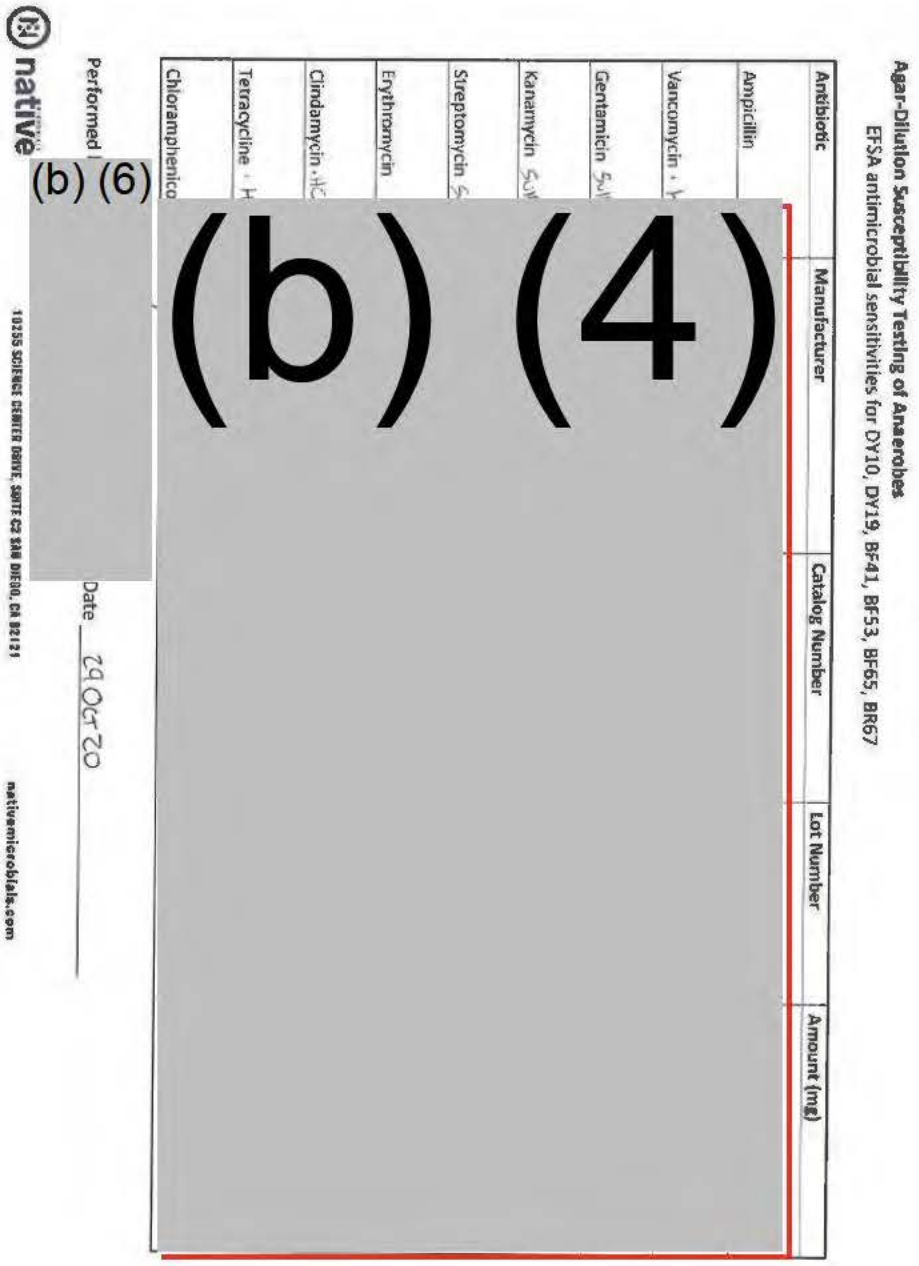
Appendix B. Raw Data

Brucella Agar

48 Project No. _____
Book No. _____ TITLE 29Oct20 Agar Dilution Media
From Page No. ____

(b) (6), (b) (4)

Antibiotic Sources



native MICROBIALS

10255 SCIENCE CENTER DRIVE, SUITE C2 SAN DIEGO, CA 92121

nativemicrobials.com

Performed by (b) (6)

Date 29 Oct 20

Agar-Dilution Susceptibility Testing of Anaerobes

EFSA antimicrobial sensitivities for DY10, DY19, BF41, BF53, BF65, BR67

Antibiotic	Manufacturer	Catalog Number	Lot Number	Amount (mg)
Ampicillin	(b) (4)			
Vancomycin · H				
Gentamicin 5o				
Kanamycin 50				
Streptomycin 5				
Erythromycin				
Clindamycin · HC				
Tetracycline · H				
Chloramphenico				

Strain Sources

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Agar-Dilution Susceptibility Testing of Anaerobes

EFSA antimicrobial sensitivities for DY10, DY19, BF41, BF53, BF65, BR67

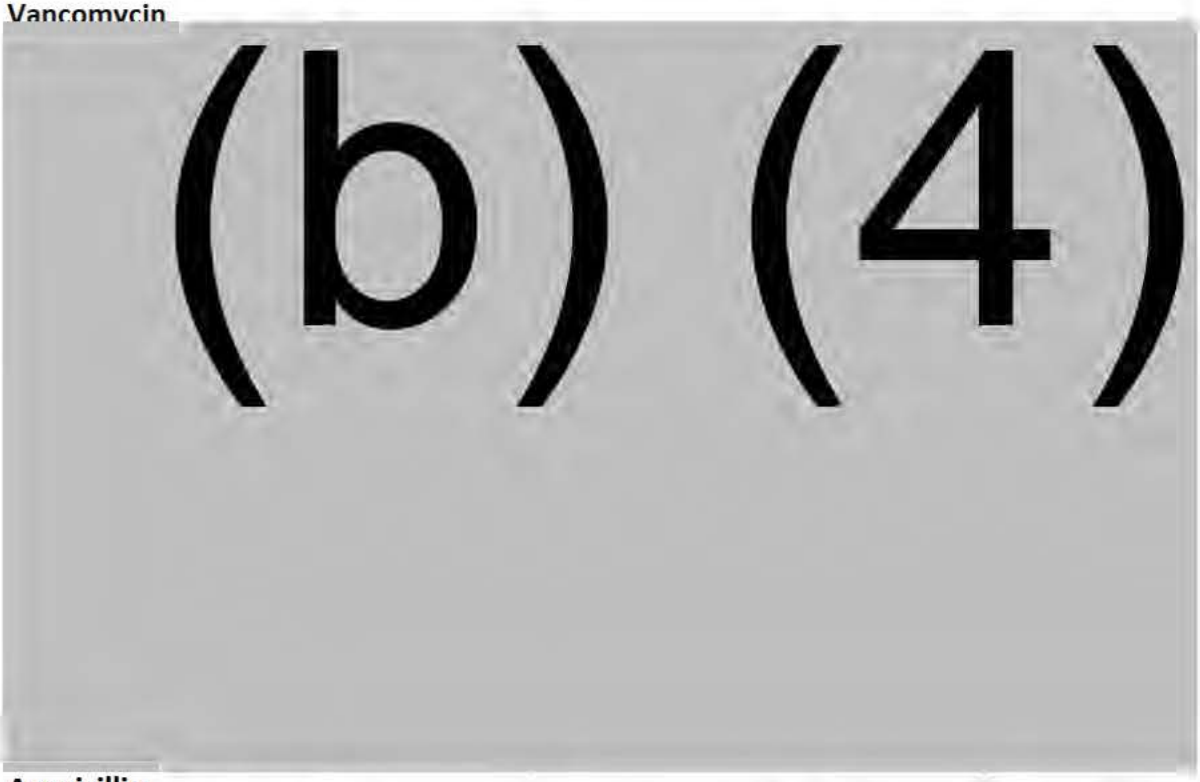
Strain (Genus species)	Strain #	Source	Inoculated (Y/N)
Ruminococcus bovis	(b) (4)		
Butyrivibrio fibrisolvens			
Prevotella albensis			
Succinivibrio dextrinosolvens			
Chondrococcus columnaris			
Clostridium beijerinckii			
Escherichia coli			

Performed by (b) (6)

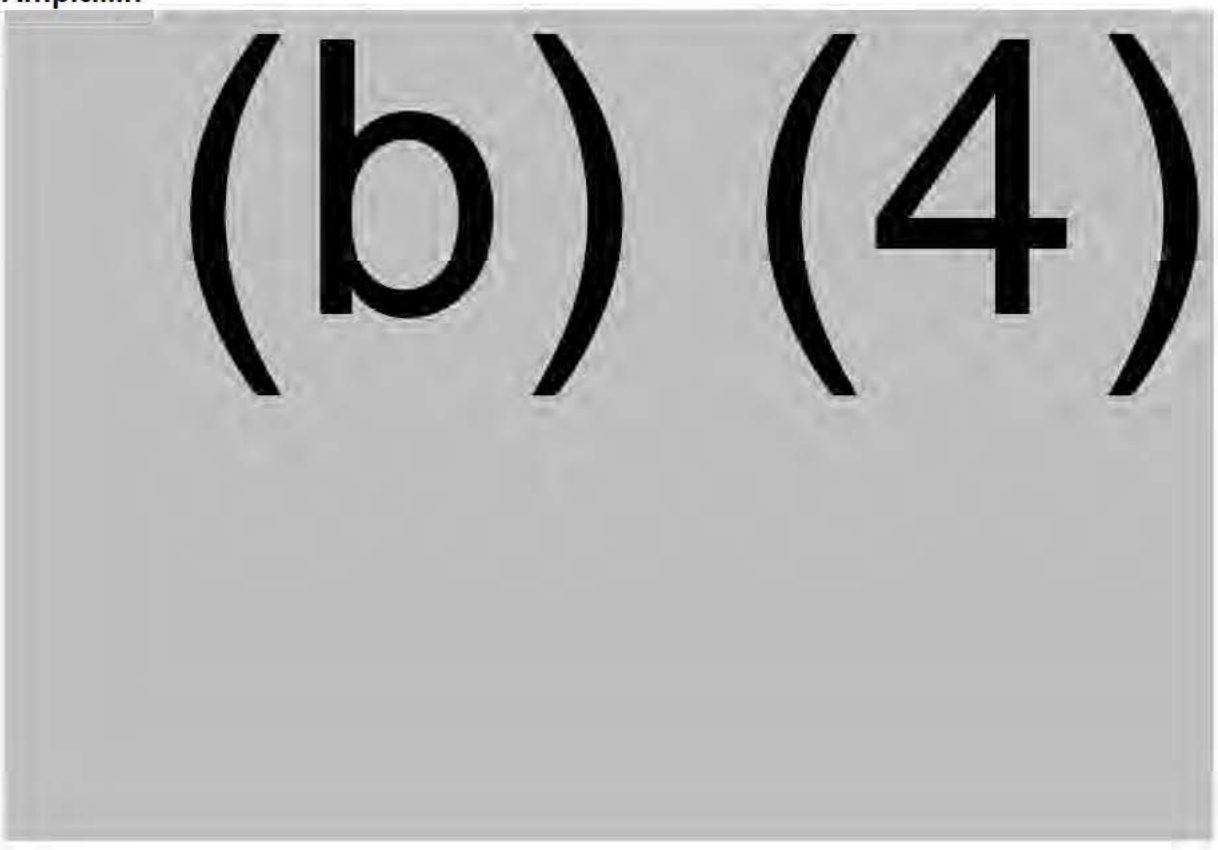
Date 30 Oct 20

Antibiotic Concentration Calculations

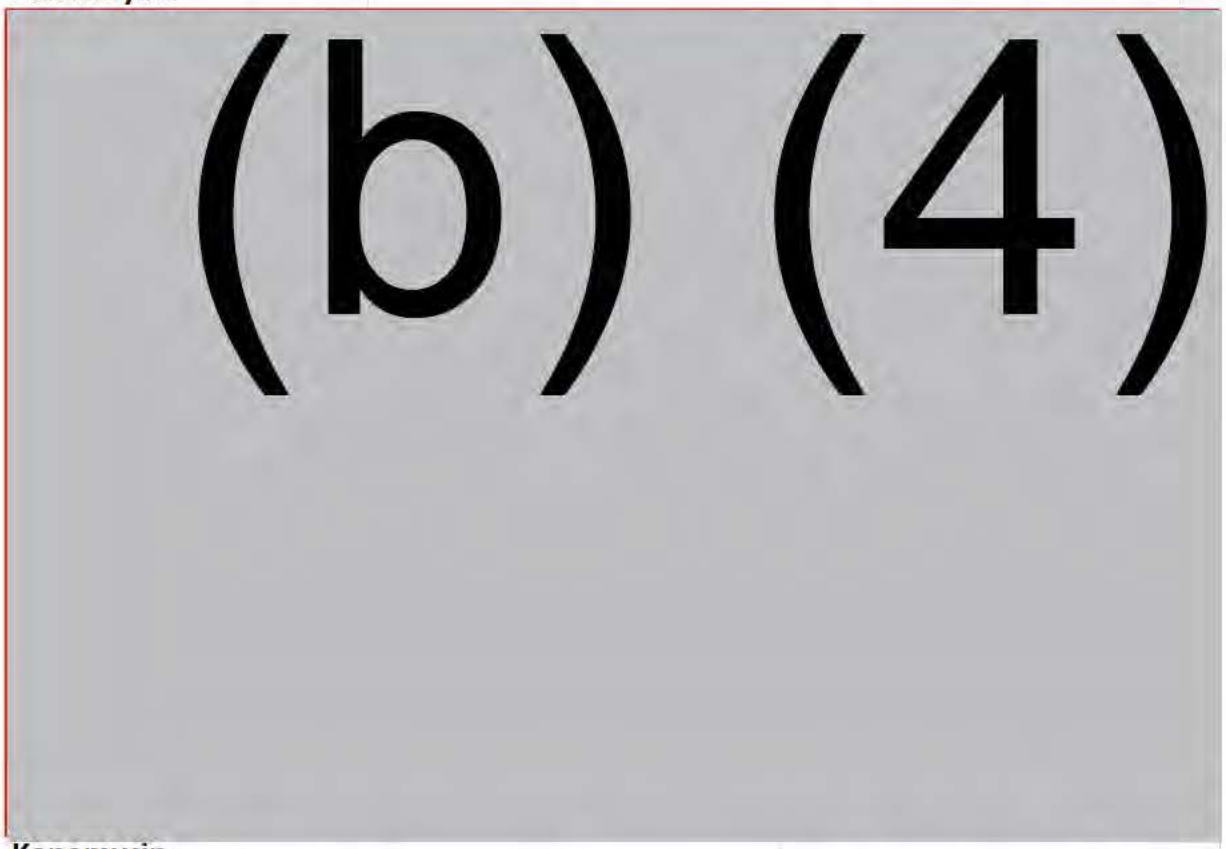
Vancomycin



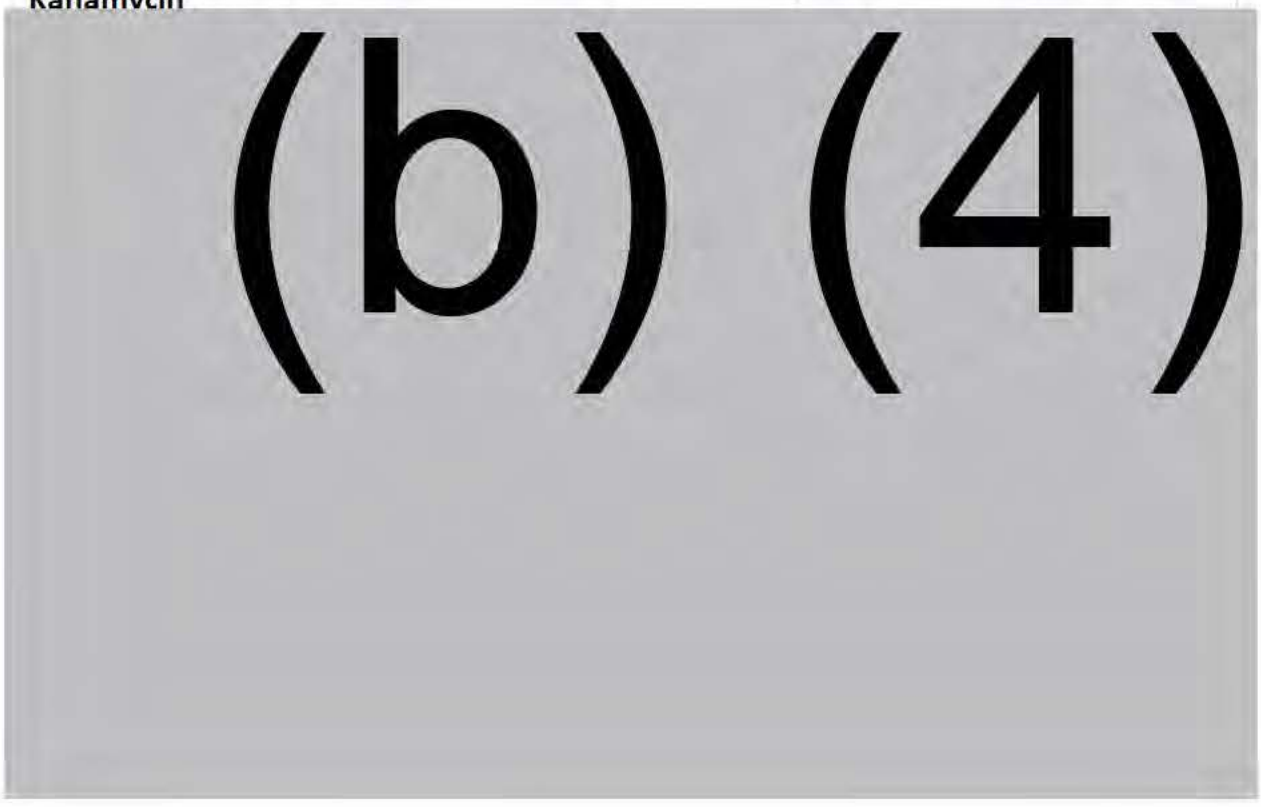
Ampicillin



Gentamycin



Kanamycin



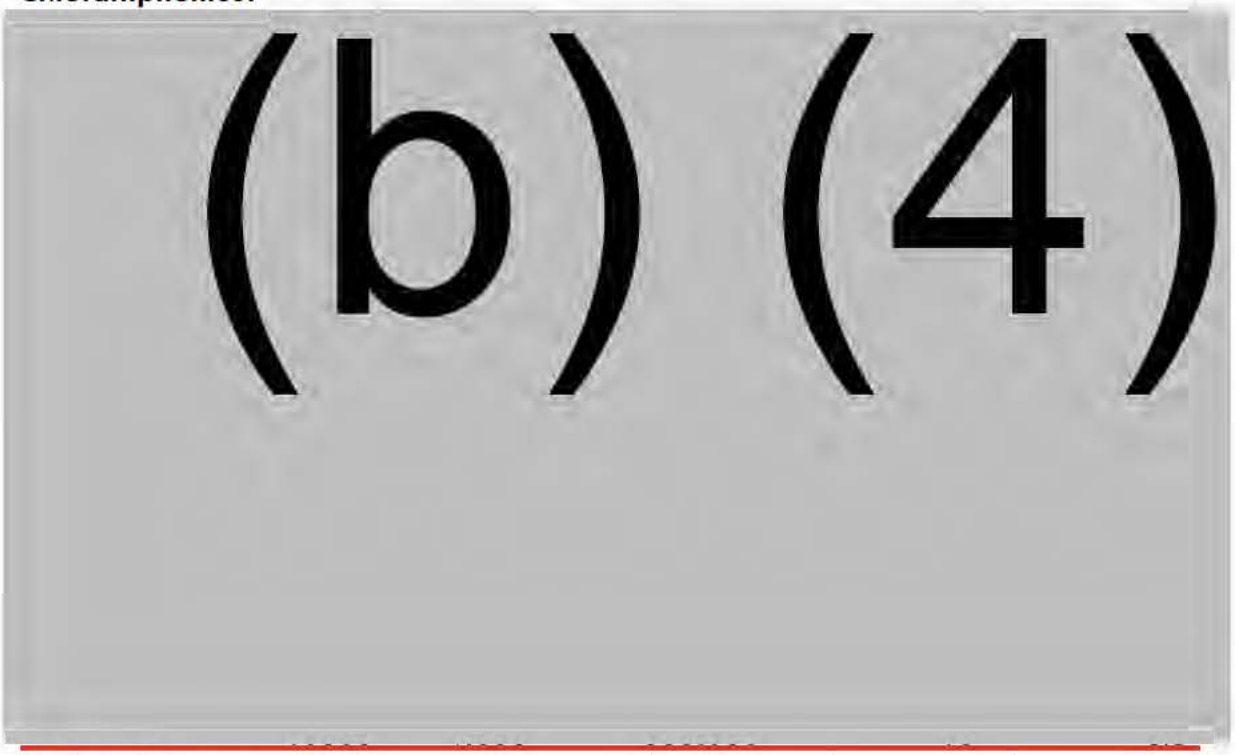
Streptomycin

(b) (4)

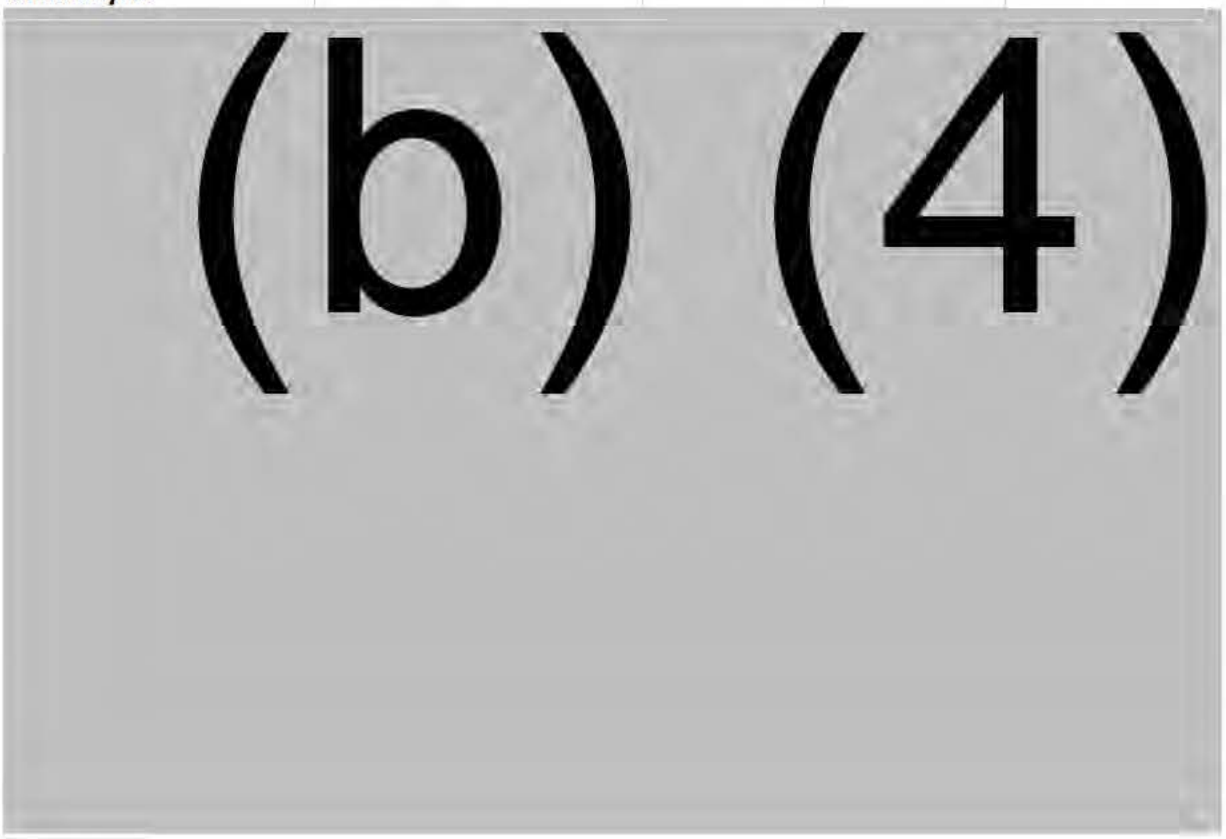
Erythromycin

(b) (4)

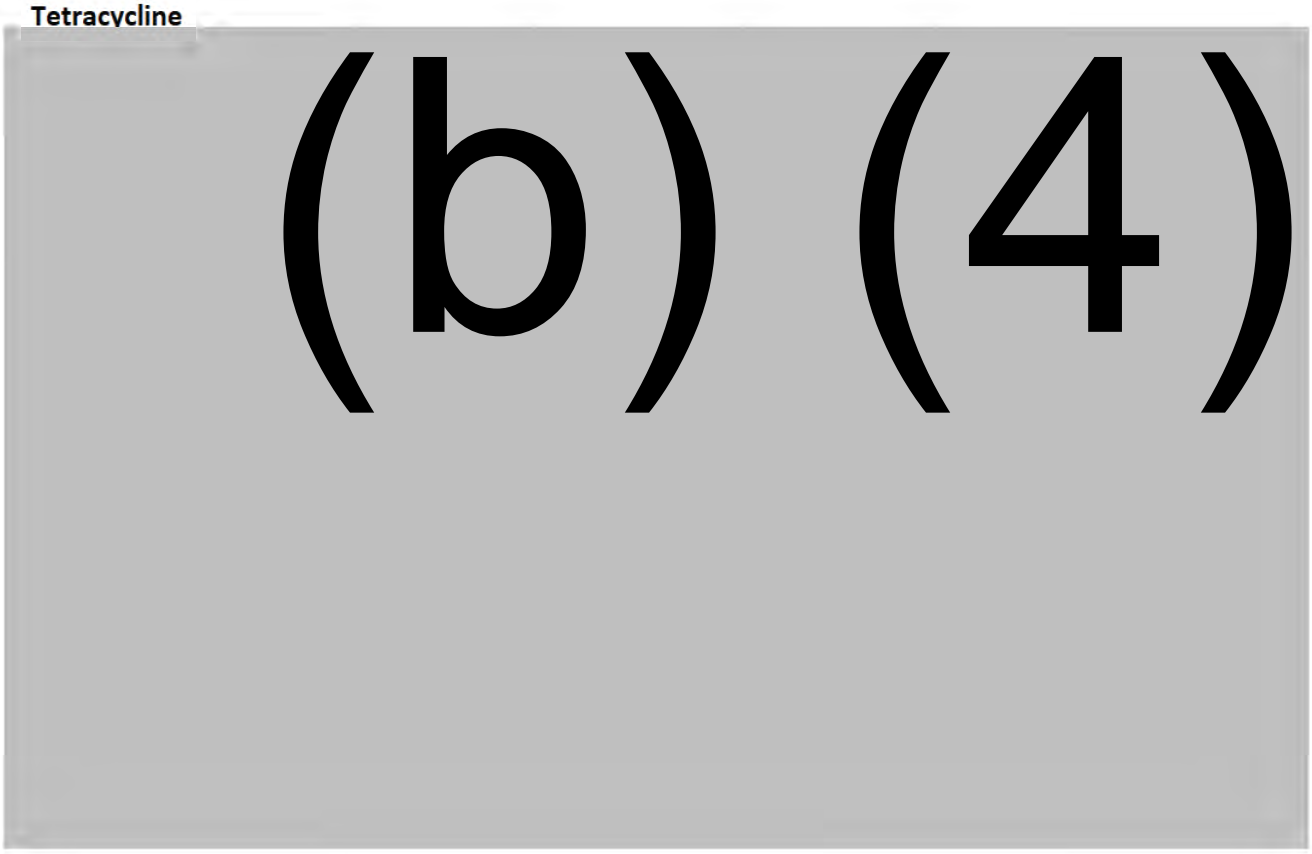
Chloramphenicol



Clindamycin



Tetracycline



Appendix C. Agar Dilution Data and Photos

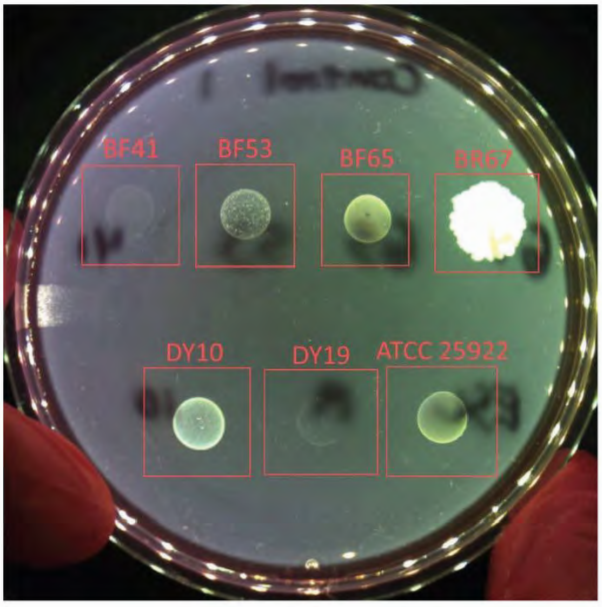
Table C-1. Agar Dilution Antibiotic Results and Susceptibility Photos: Ampicillin

	Ampicillin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	NG	NG	NG	NG	NG	NG	NG
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	NG	NG	NG	NG	NG	NG

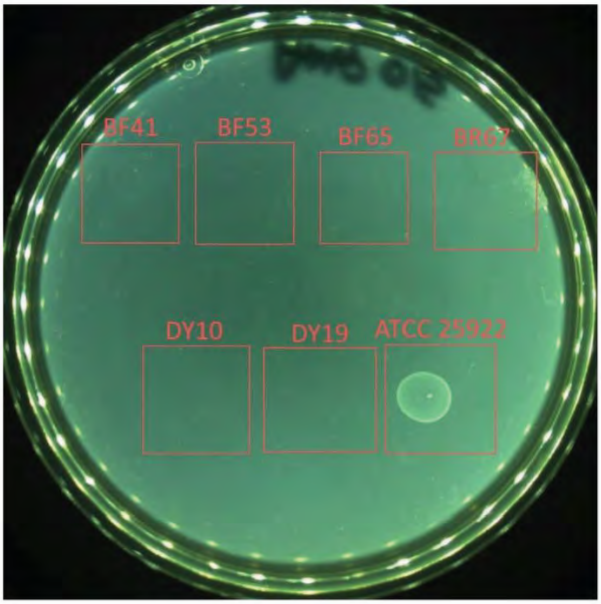
G = Growth

NG = No Growth

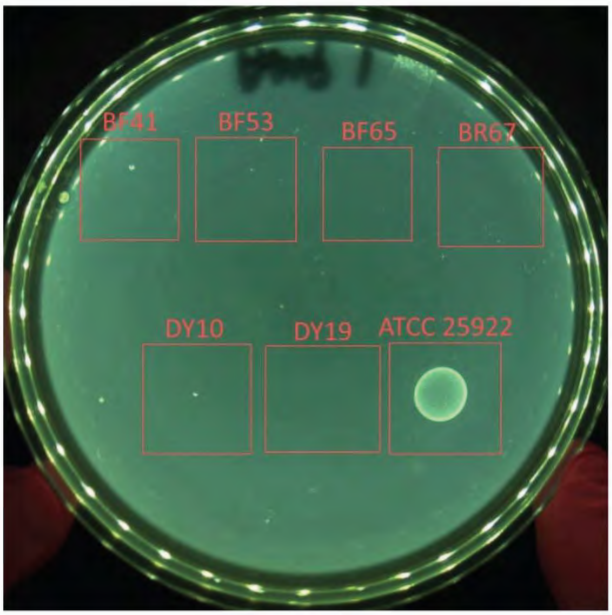
Agar Dilution Antibiotic Susceptibility Photos: Ampicillin



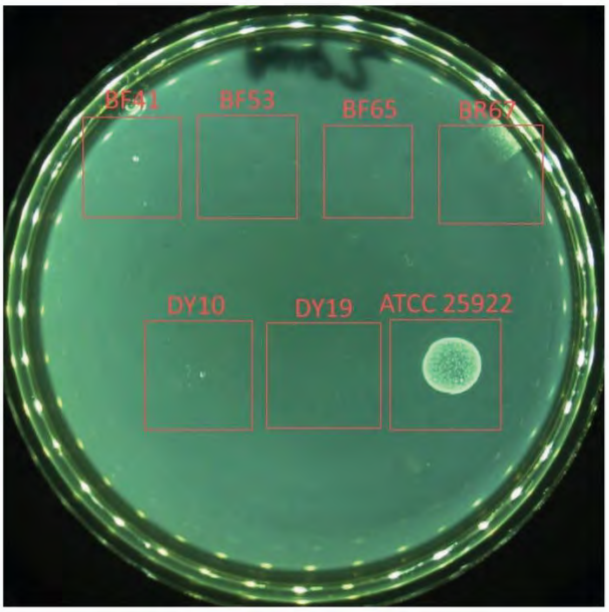
0 µg/mL Ampicillin



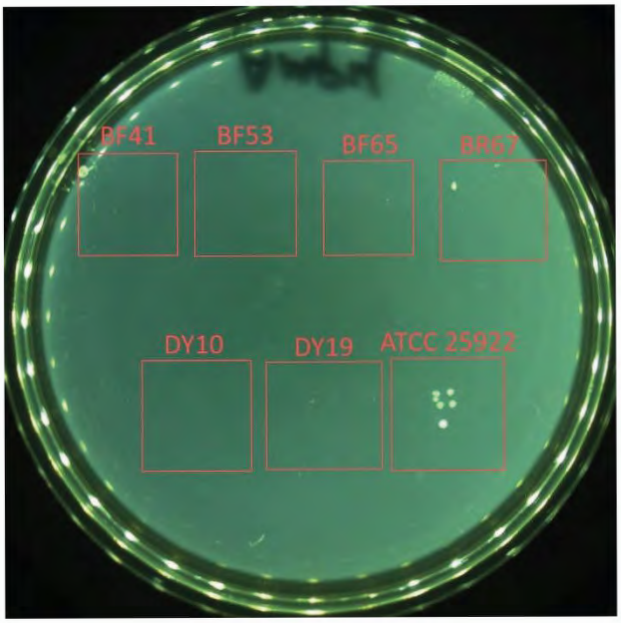
0.5 µg/mL Ampicillin



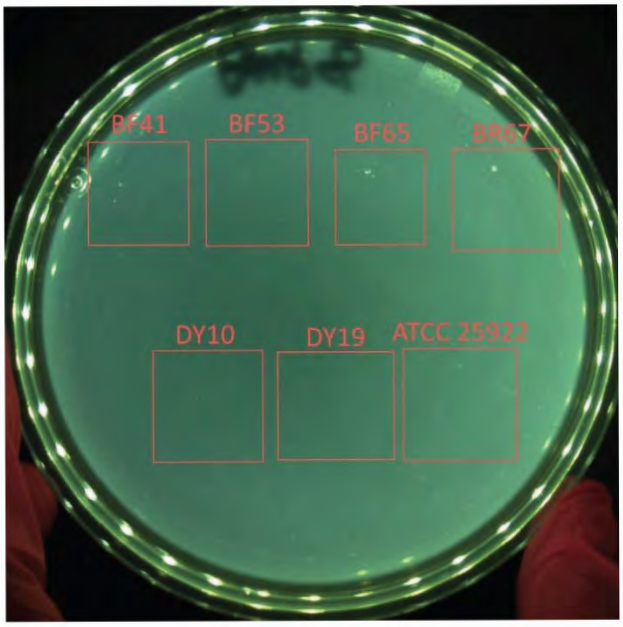
1 µg/mL Ampicillin



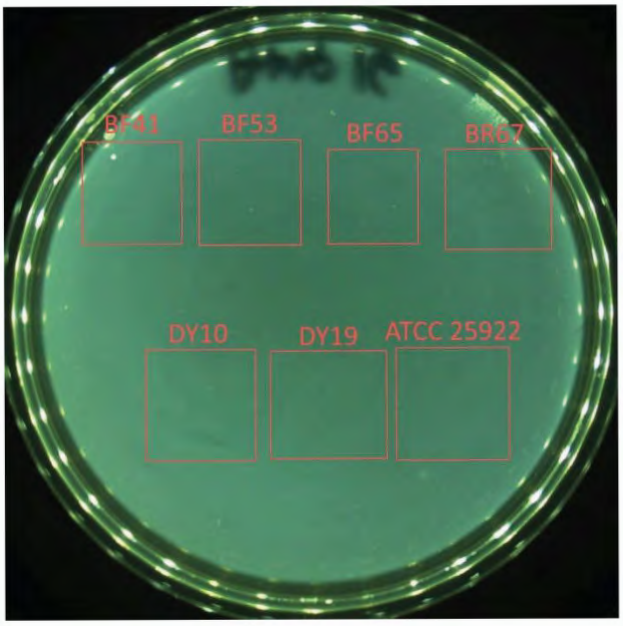
2 µg/mL Ampicillin



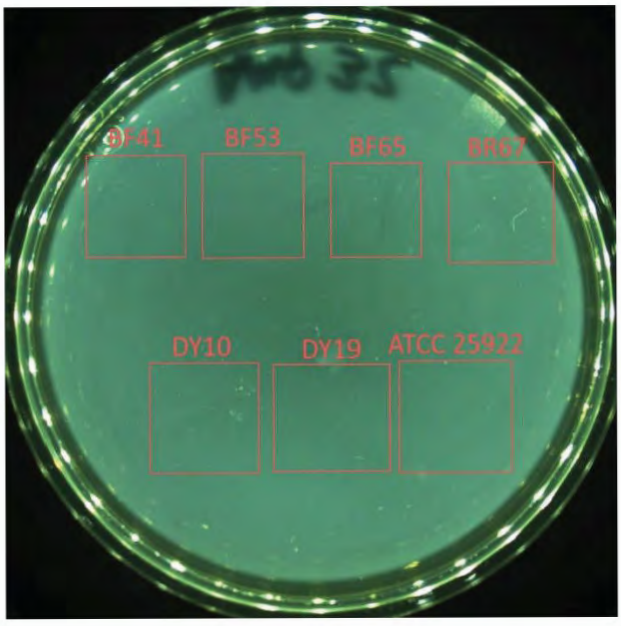
4 µg/mL Ampicillin



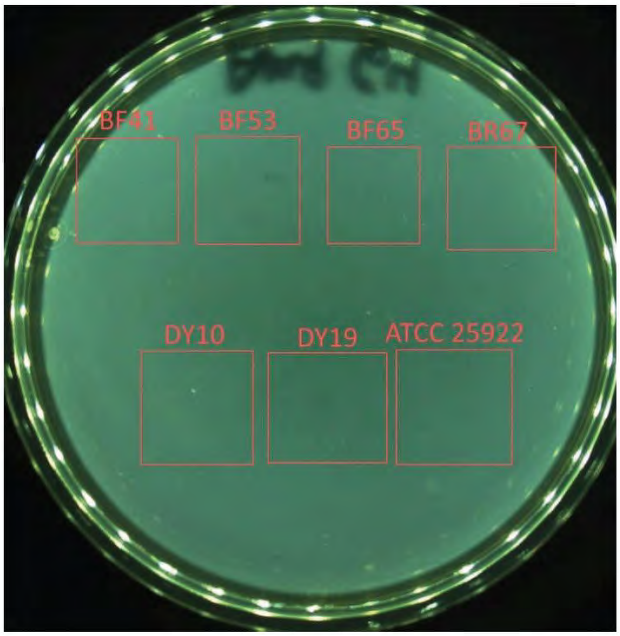
8 µg/mL Ampicillin



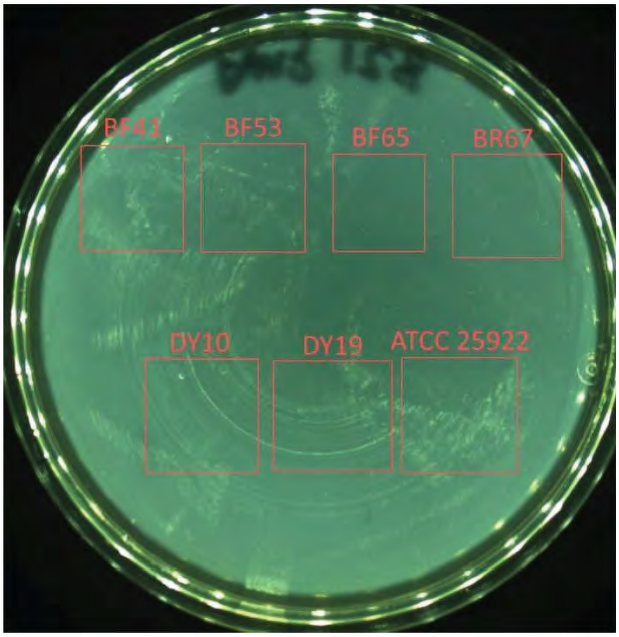
16 µg/mL Ampicillin



32 µg/mL Ampicillin



64 µg/ml Ampicillin



128 µg/ml Ampicillin

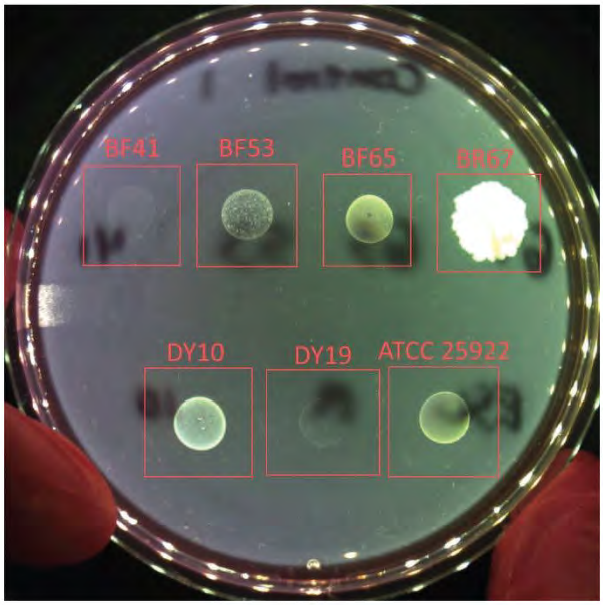
Table C-2. Agar Dilution Antibiotic Results and Susceptibility Photos: Chloramphenicol

	Chloramphenicol Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	NG	NG	NG	NG

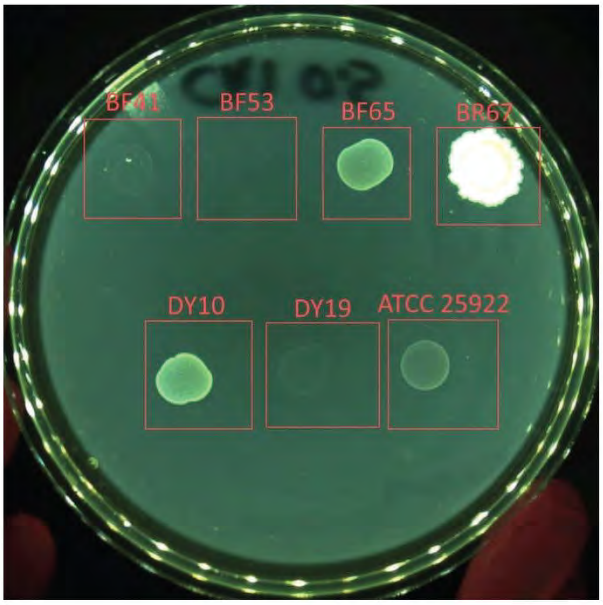
G = Growth

NG = No Growth

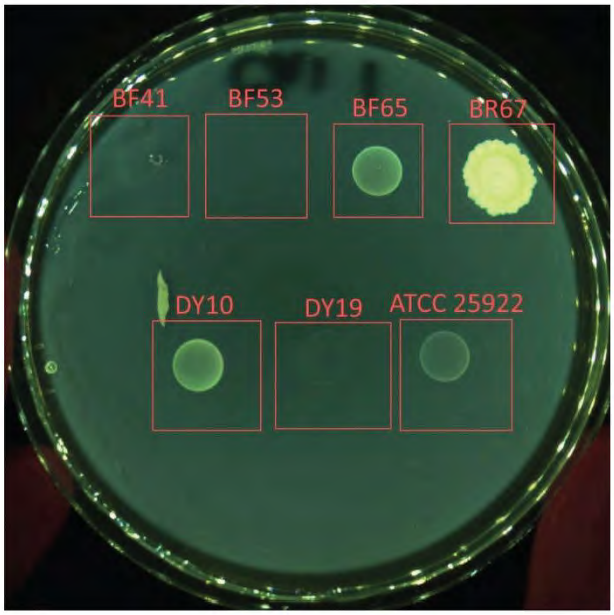
Agar Dilution Antibiotic Susceptibility Photos: Chloramphenicol



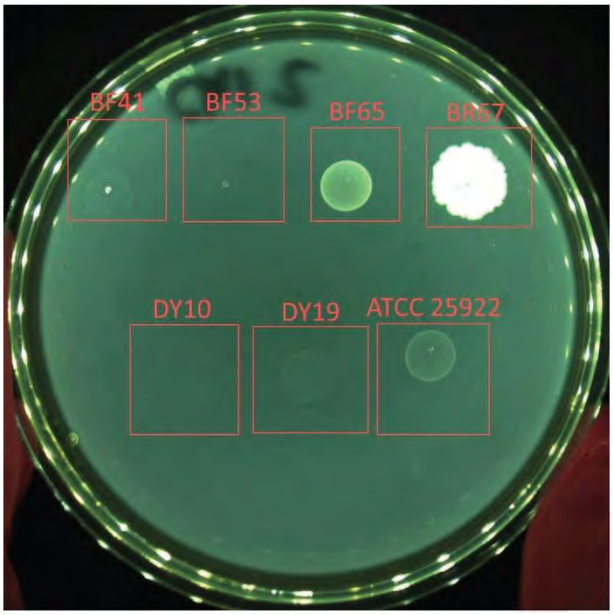
0 µg/mL Chloramphenicol



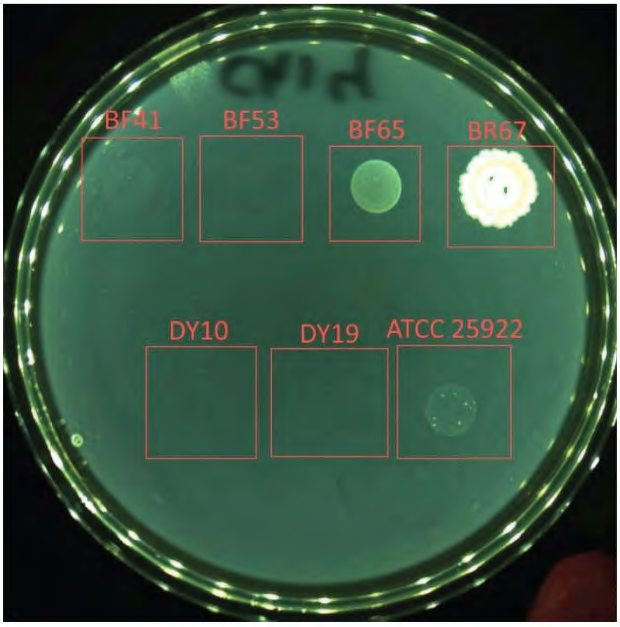
0.5 µg/mL Chloramphenicol



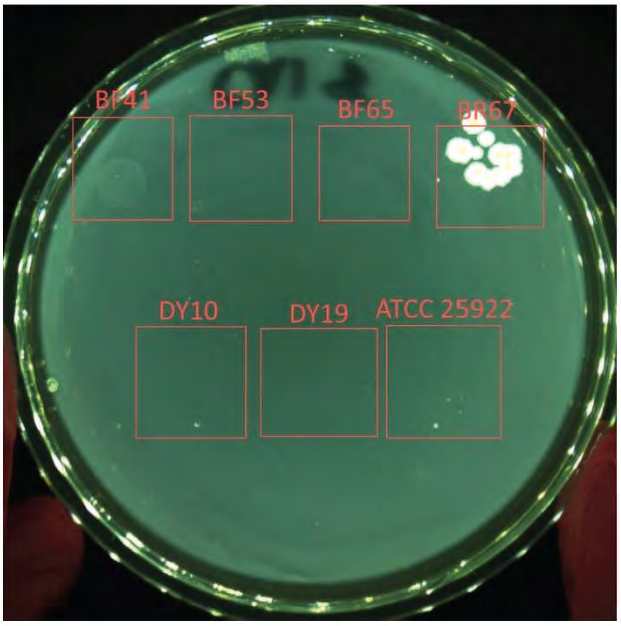
1 µg/mL Chloramphenicol



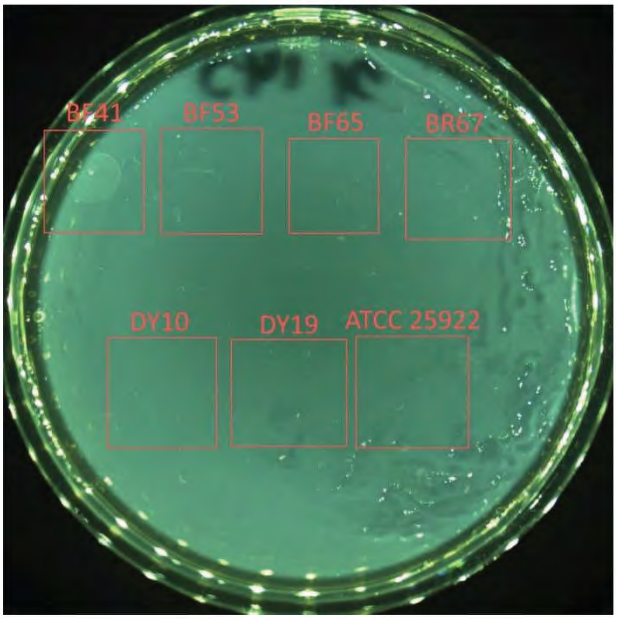
2 µg/mL Chloramphenicol



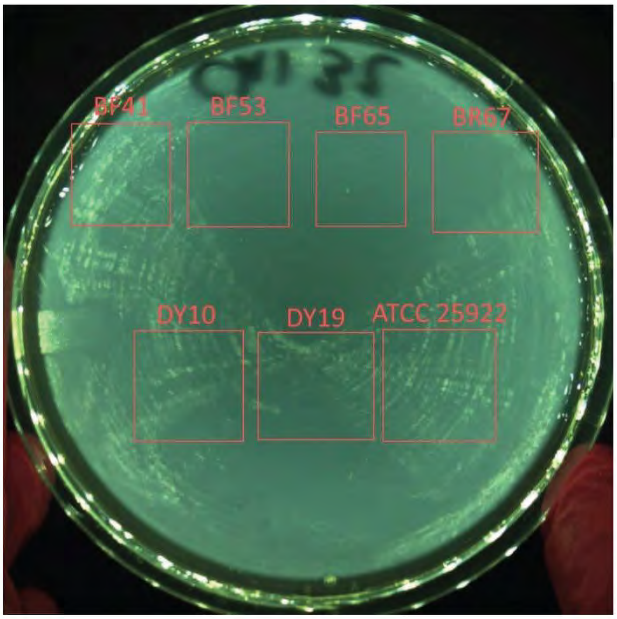
4 µg/mL Chloramphenicol



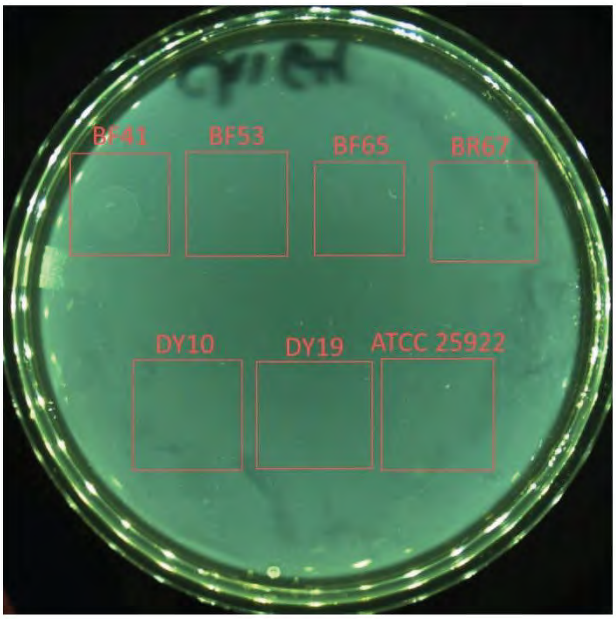
8 µg/mL Chloramphenicol



16 µg/mL Chloramphenicol



32 µg/mL Chloramphenicol



64 µg/ml Chloramphenicol

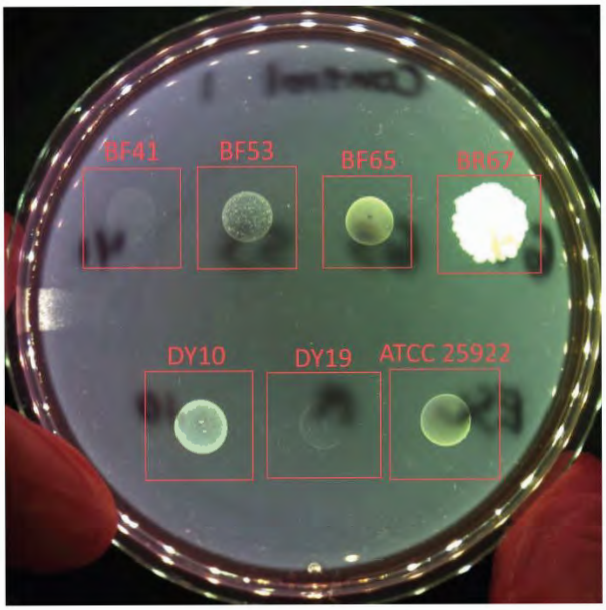
Table C-3. Agar Dilution Antibiotic Results and Susceptibility Photos: Clindamycin

	Clindamycin Concentration (µg/mL)											
Organism	**0 (Control)**	**0.03125**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G	G	G

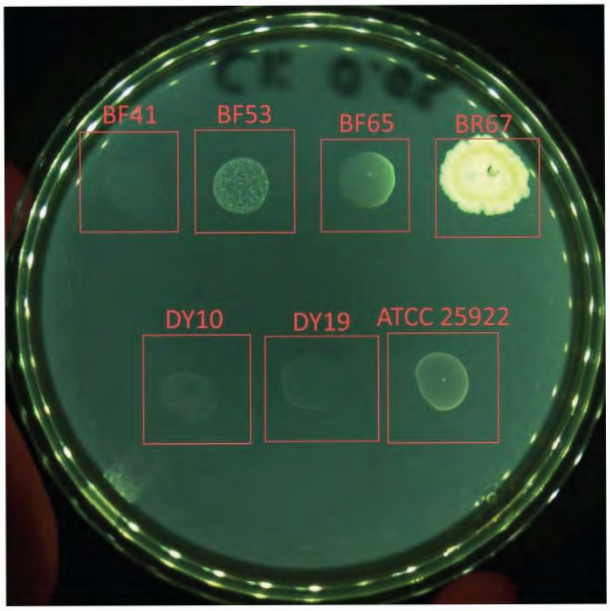
G = Growth

NG = No Growth

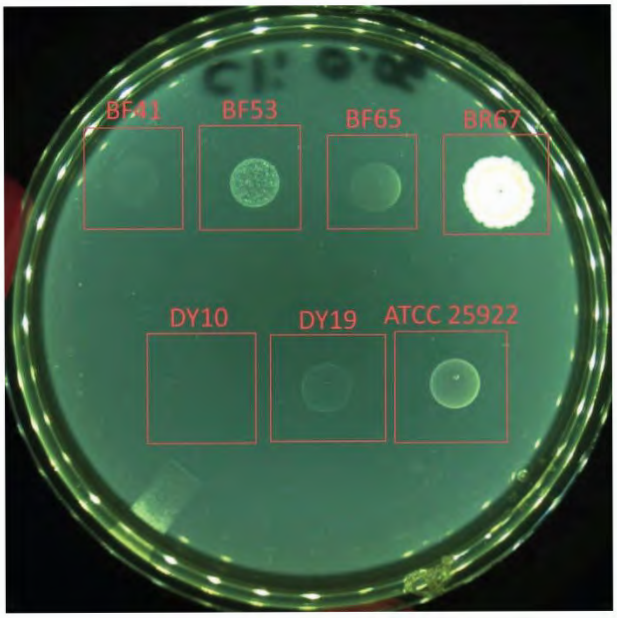
Agar Dilution Antibiotic Susceptibility Photos: Clindamycin



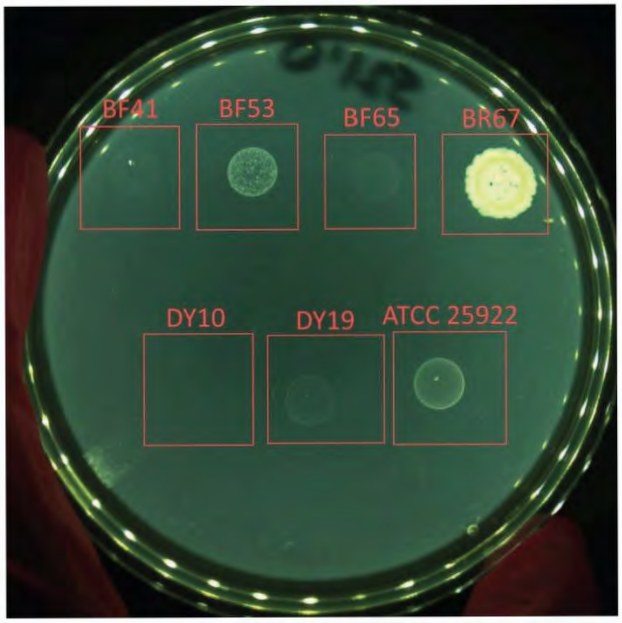
0 µg/mL Clindamycin



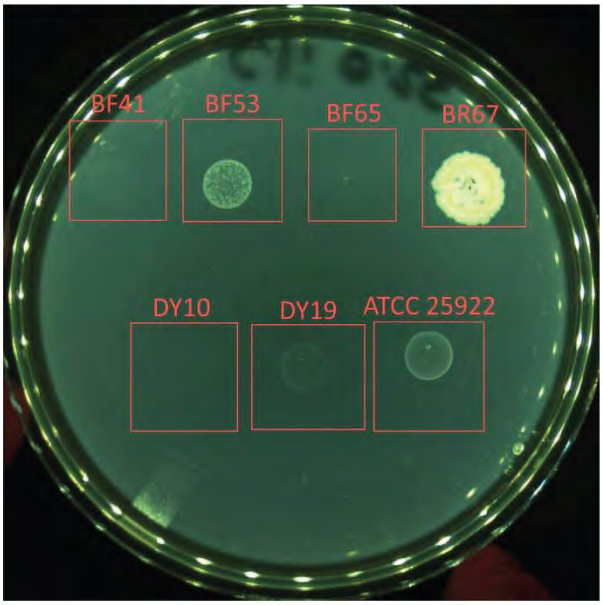
0.03125 µg/mL Clindamycin



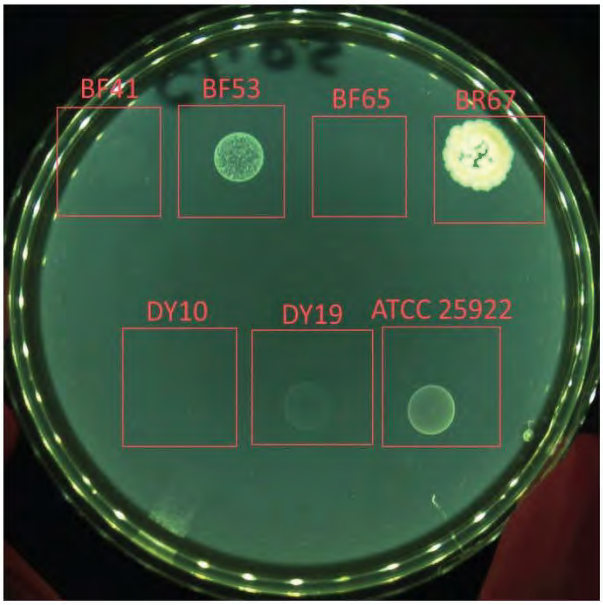
0.0625 µg/mL Clindamycin



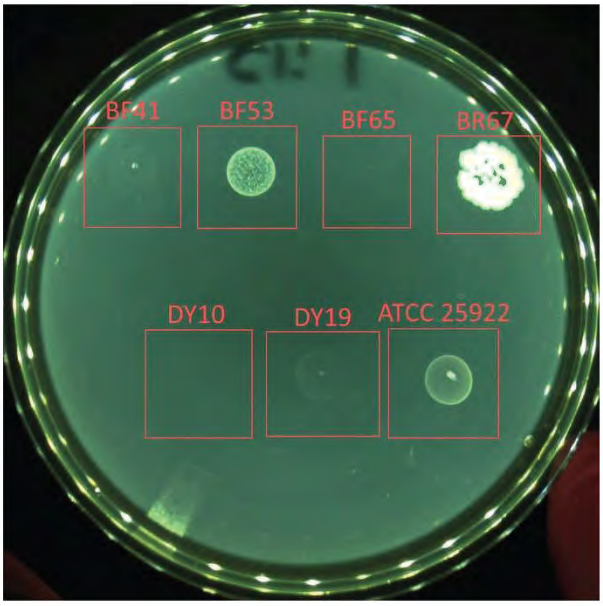
0.125 µg/mL Clindamycin



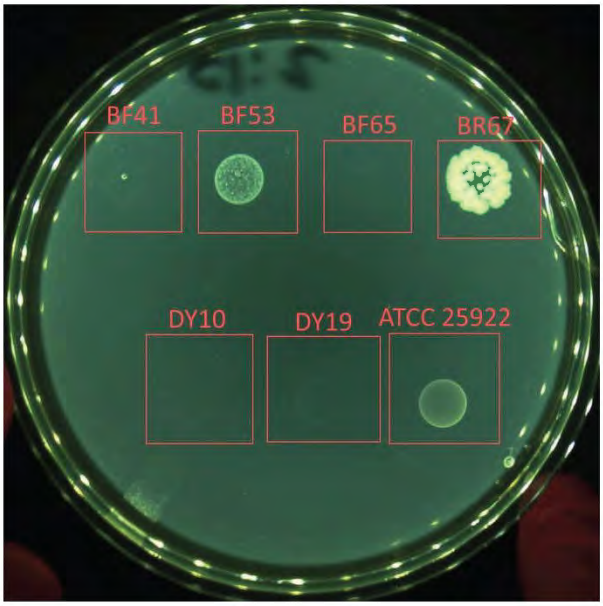
0.25 µg/mL Clindamycin



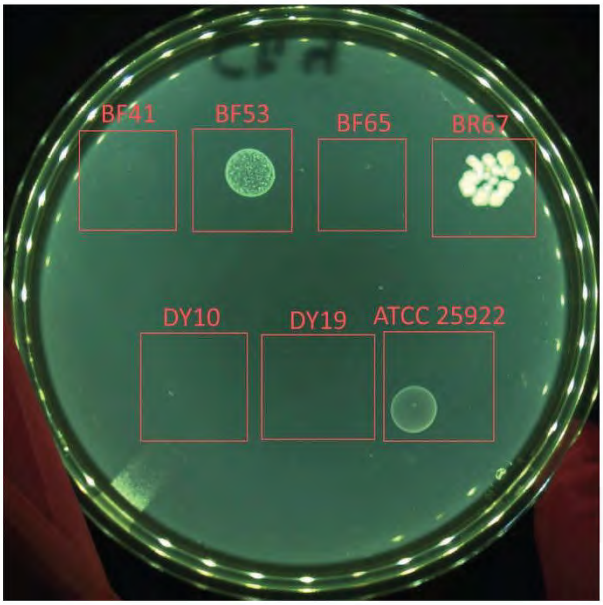
0.5 µg/mL Clindamycin



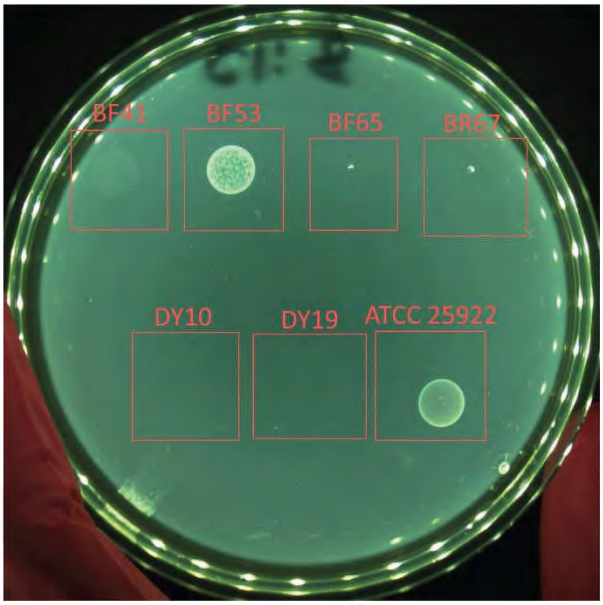
1 µg/mL Clindamycin



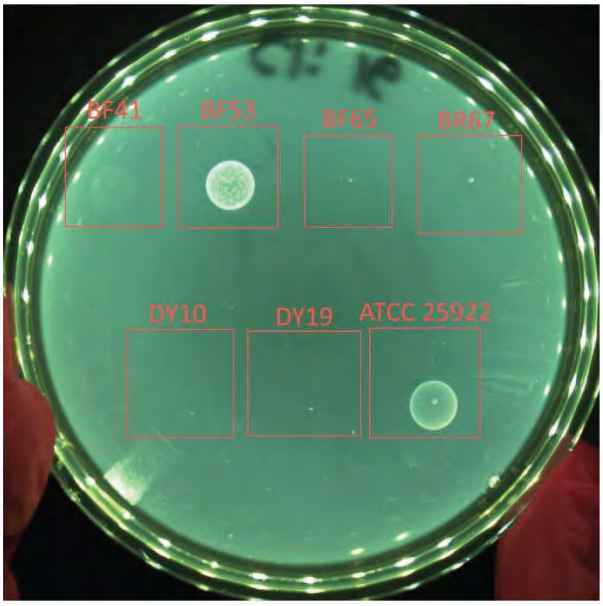
2 µg/mL Clindamycin



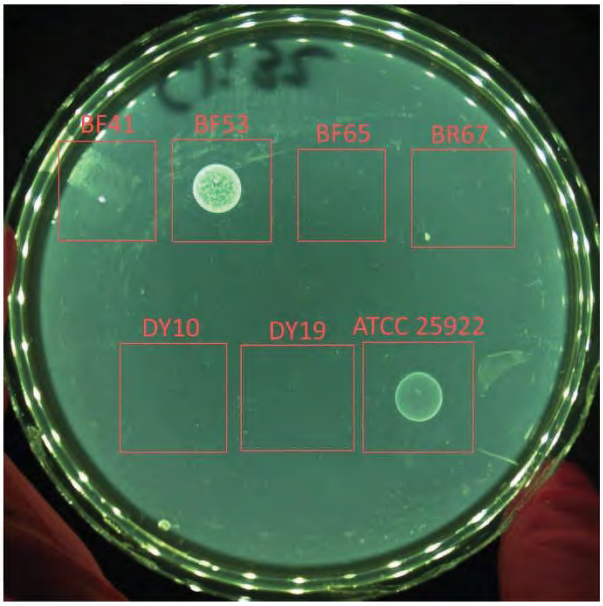
4 µg/mL Clindamycin



8 µg/mL Clindamycin



16 µg/mL Clindamycin



32 µg/mL Clindamycin

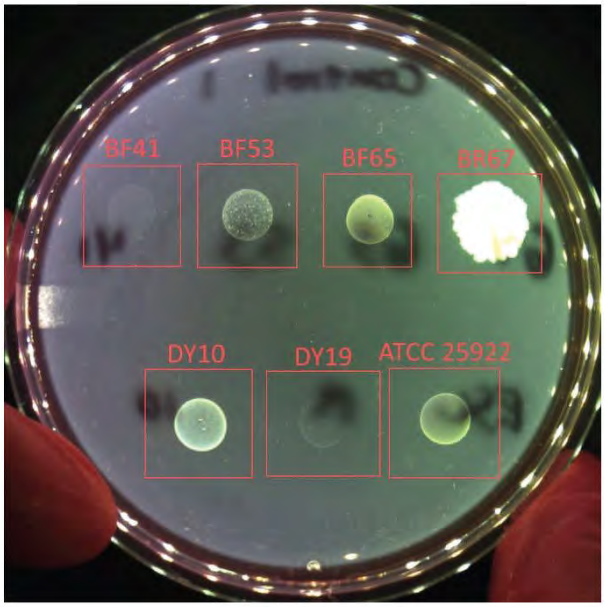
Table C-4. Agar Dilution Antibiotic Results and Susceptibility Photos: Erythromycin

	Erythromycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

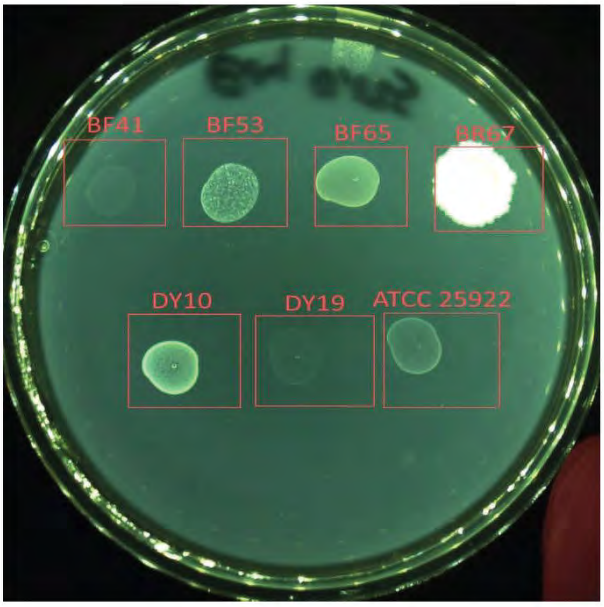
G = Growth

NG = No Growth

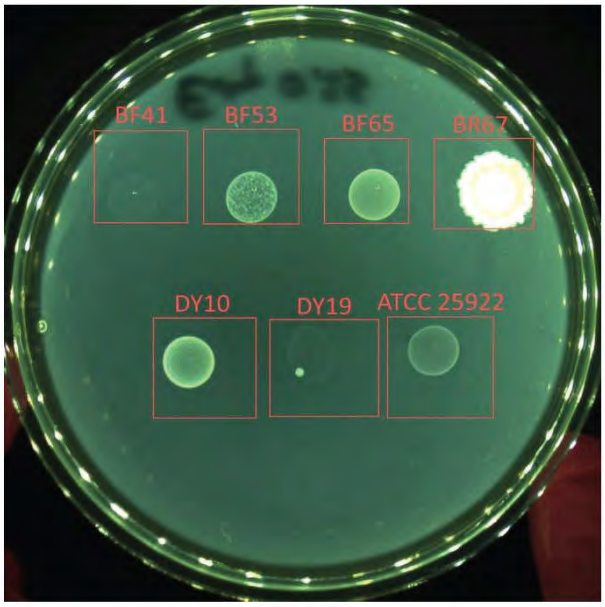
Agar Dilution Antibiotic Susceptibility Photos: Erythromycin



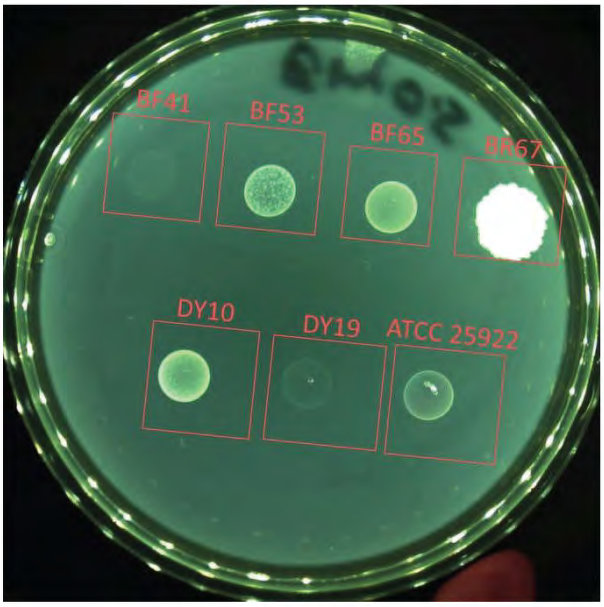
0 µg/mL Erythromycin



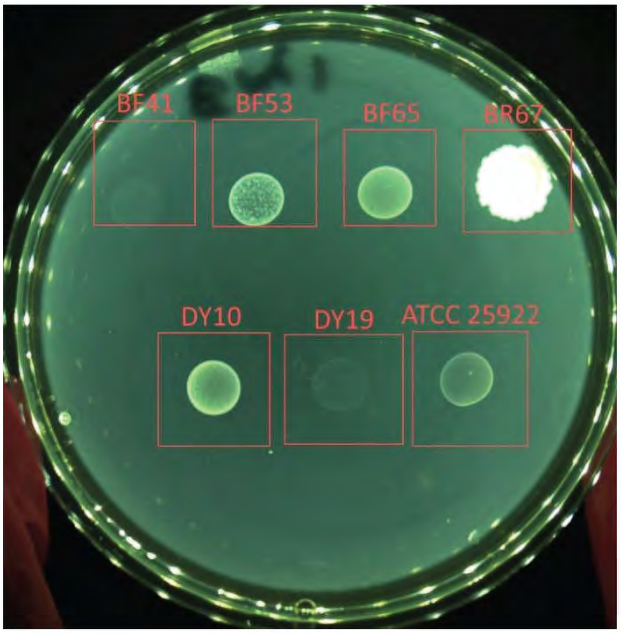
0.125 µg/mL Erythromycin



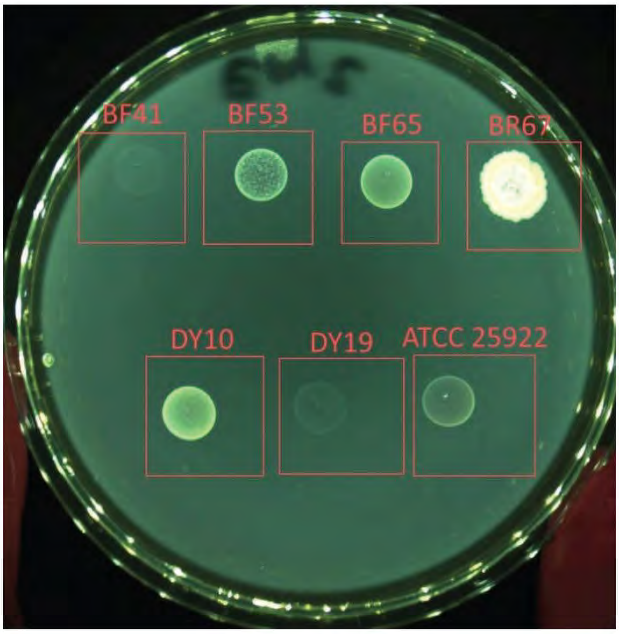
0.25 µg/mL Erythromycin



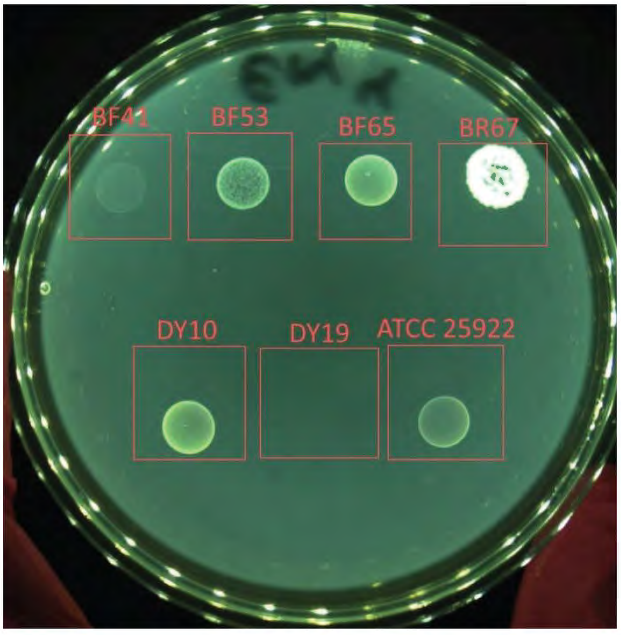
0.5 µg/mL Erythromycin



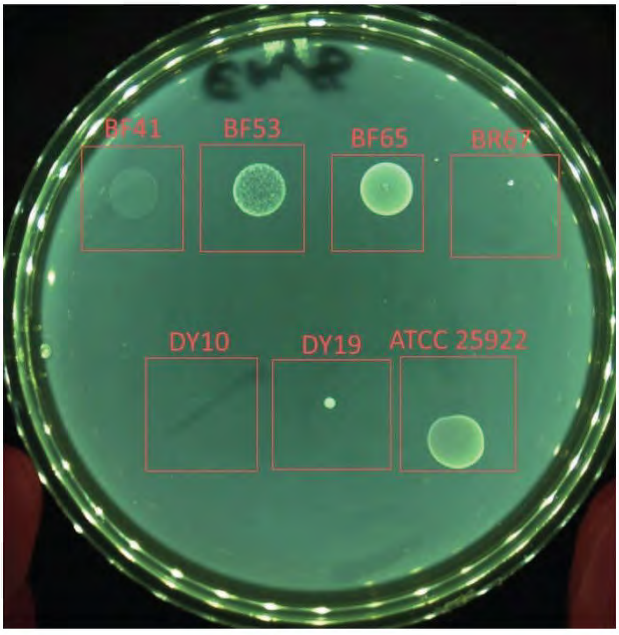
1 µg/mL Erythromycin



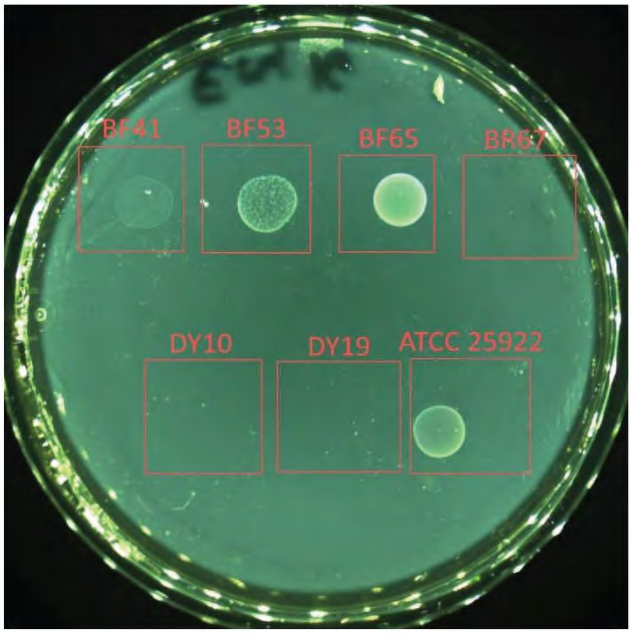
2 µg/mL Erythromycin



4 µg/mL Erythromycin



8 µg/mL Erythromycin



16 µg/ml Erythromycin

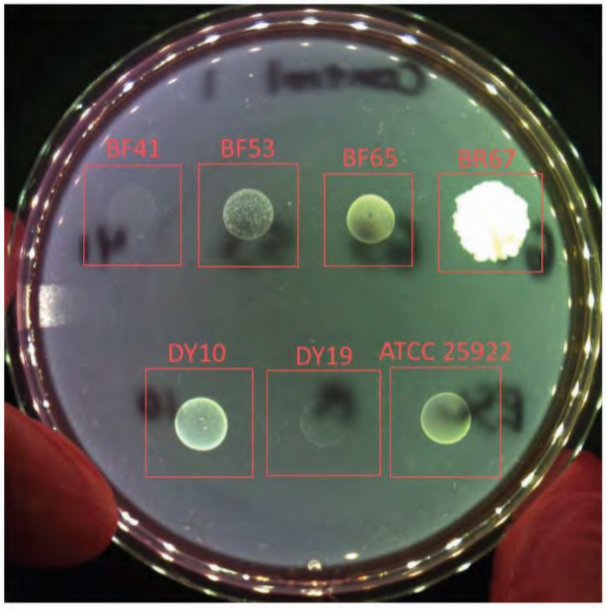
Table C-5. Agar Dilution Antibiotic Results and Susceptibility Photos: Gentamicin

	Gentamicin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G	G
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G

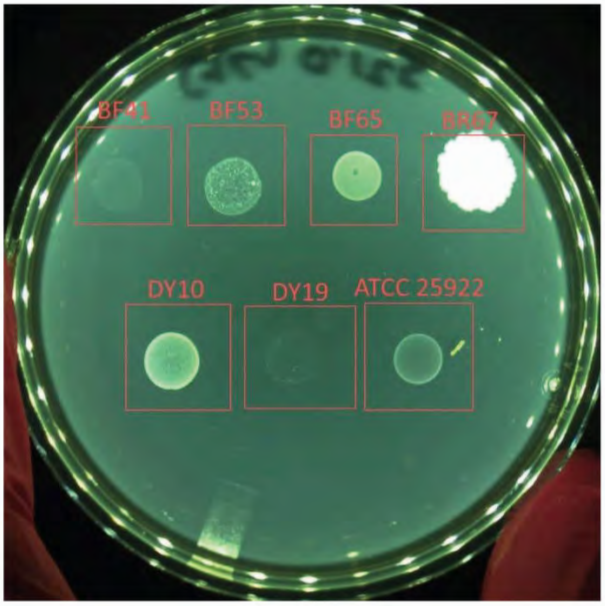
G = Growth

NG = No Growth

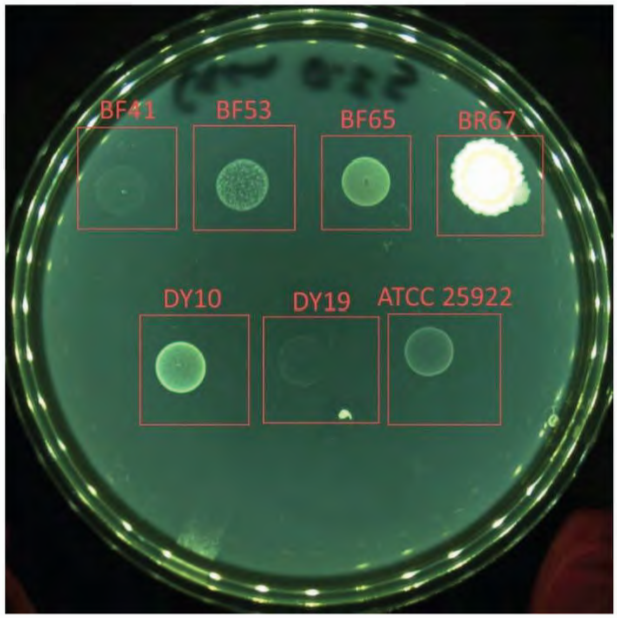
Agar Dilution Antibiotic Susceptibility Photos: Gentamicin



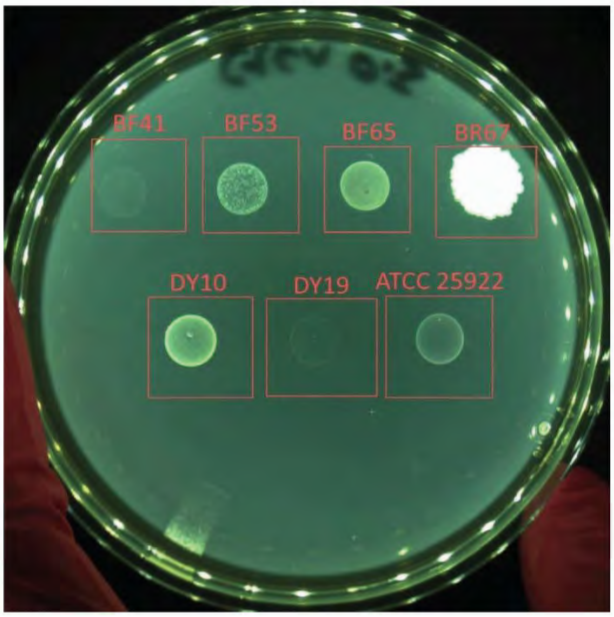
0 µg/mL Gentamicin



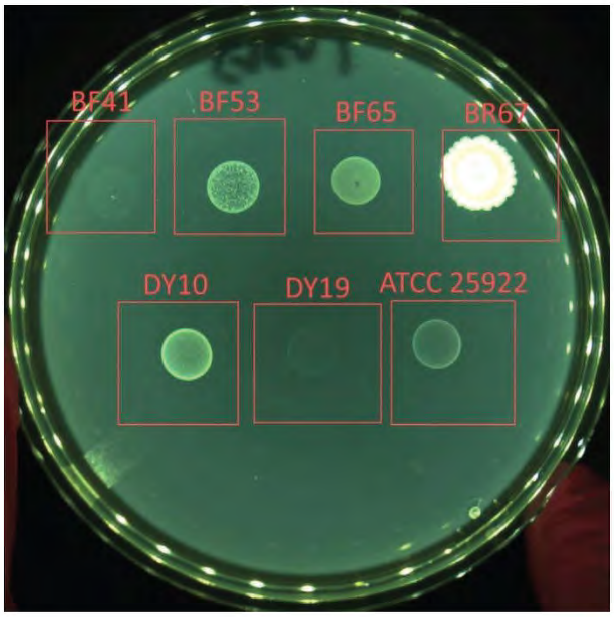
0.125 µg/mL Gentamicin



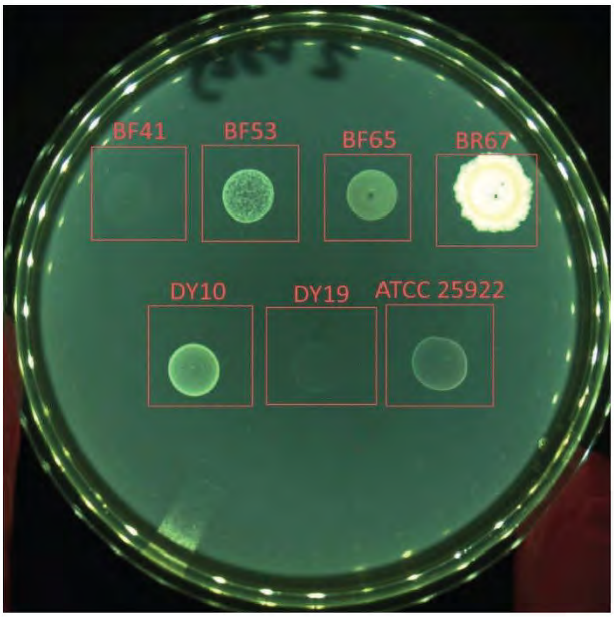
0.25 µg/mL Gentamicin



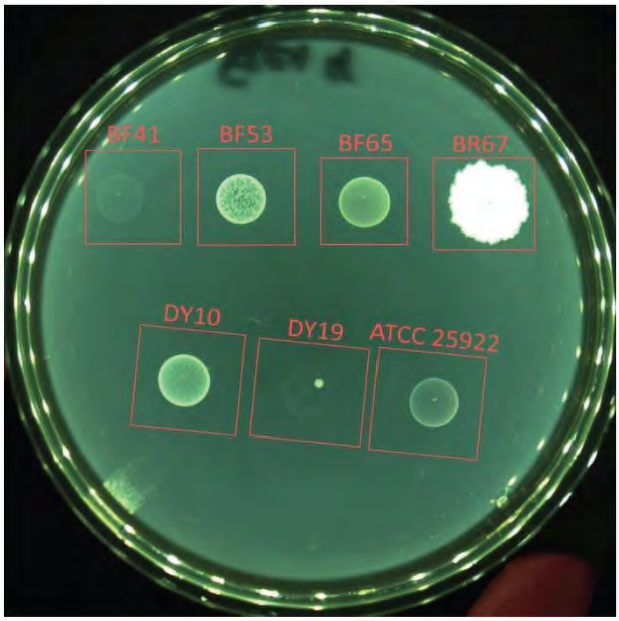
0.5 µg/mL Gentamicin



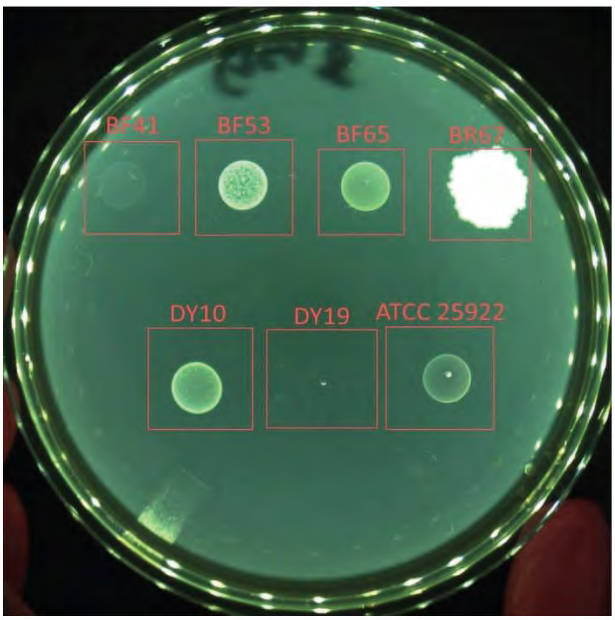
1 µg/mL Gentamicin



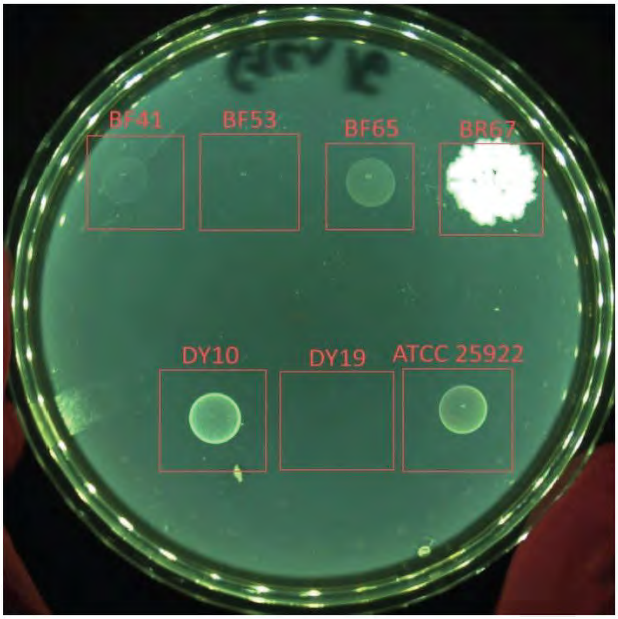
2 µg/mL Gentamicin



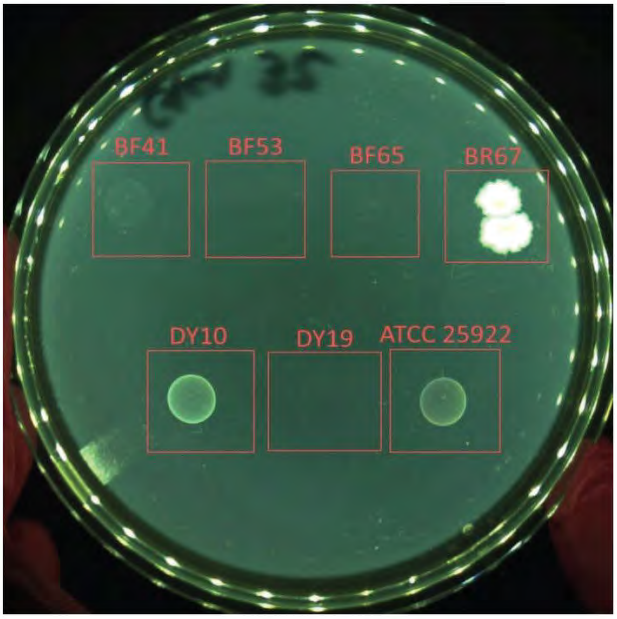
4 µg/mL Gentamicin



8 µg/mL Gentamicin



16 µg/mL Gentamicin



32 µg/mL Gentamicin

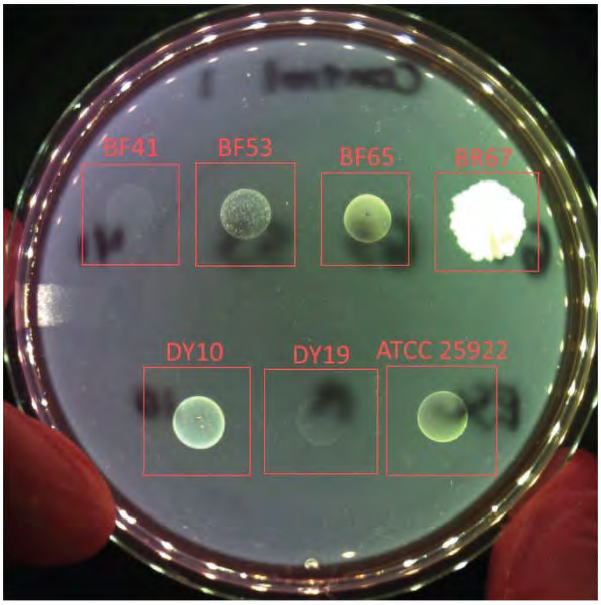
Table C-6. Agar Dilution Antibiotic Results and Susceptibility Photos: Kanamycin

	Kanamycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	G
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

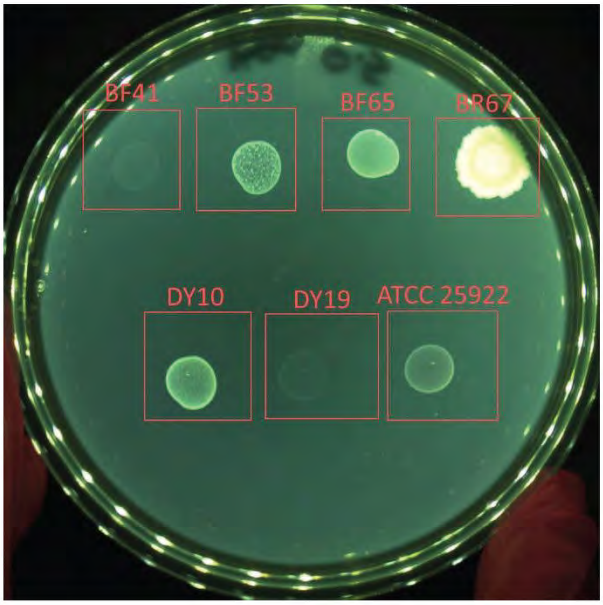
G = Growth

NG = No Growth

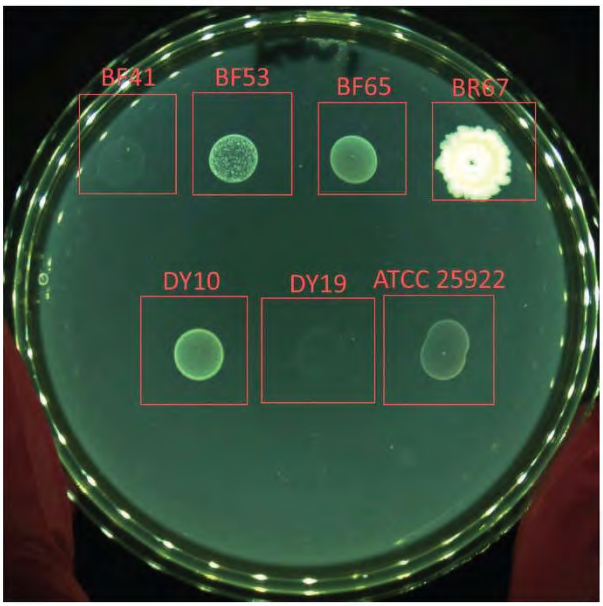
Agar Dilution Antibiotic Susceptibility Photos: Kanamycin



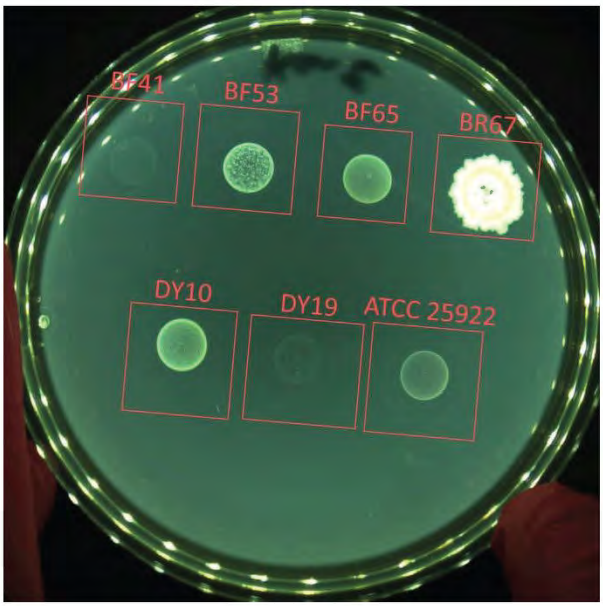
0 µg/mL Kanamycin



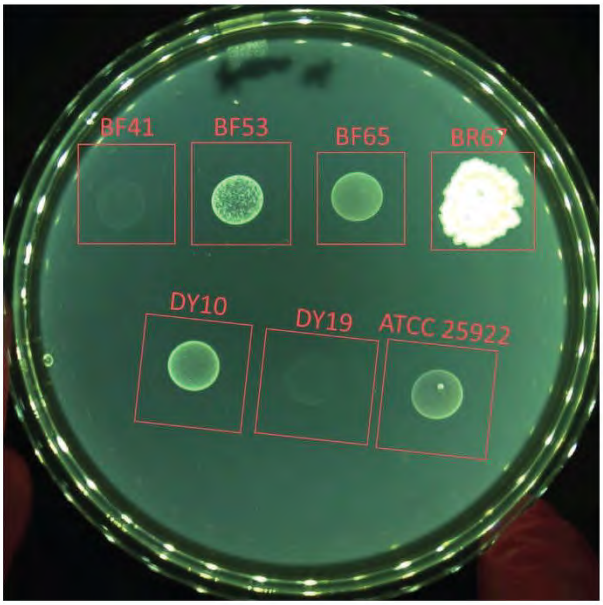
0.5 µg/mL Kanamycin



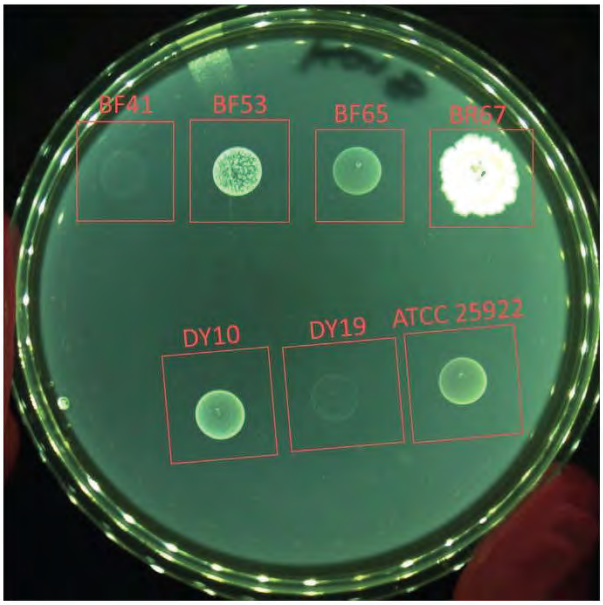
1 µg/mL Kanamycin



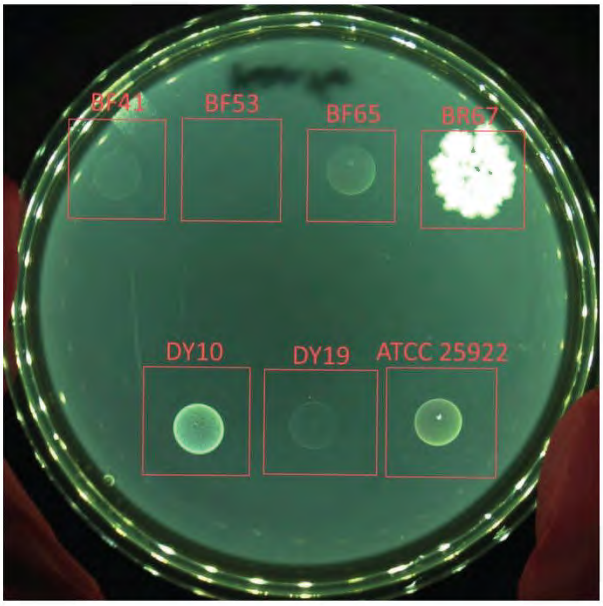
2 µg/mL Kanamycin



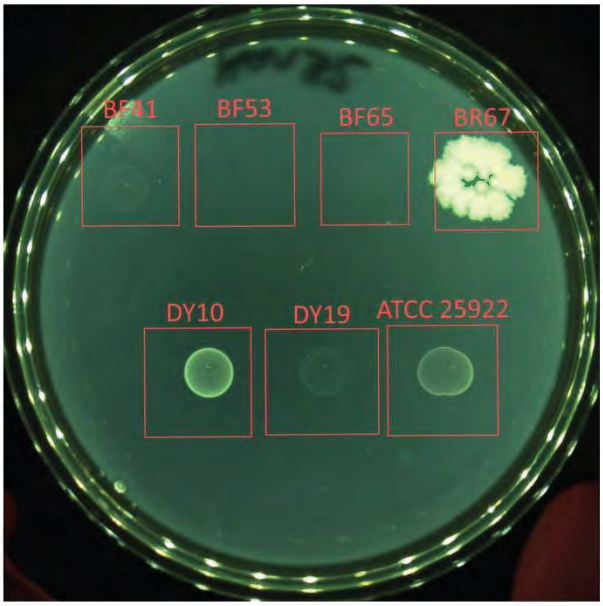
4 µg/mL Kanamycin



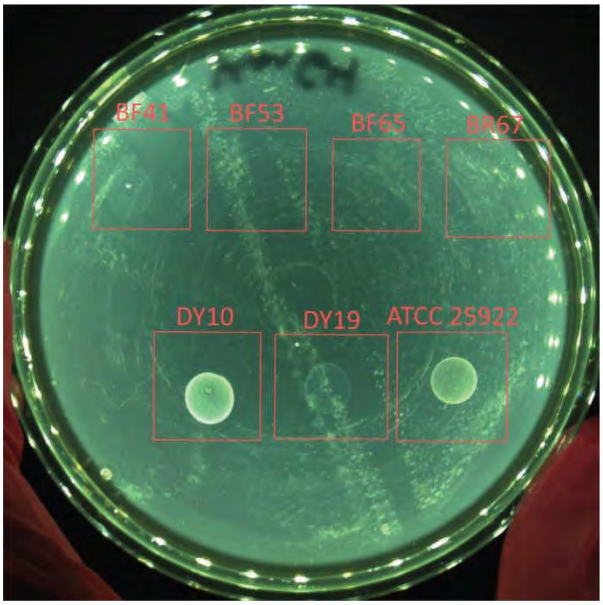
8 µg/mL Kanamycin



16 µg/mL Kanamycin



32 µg/mL Kanamycin



64 µg/mL Kanamycin

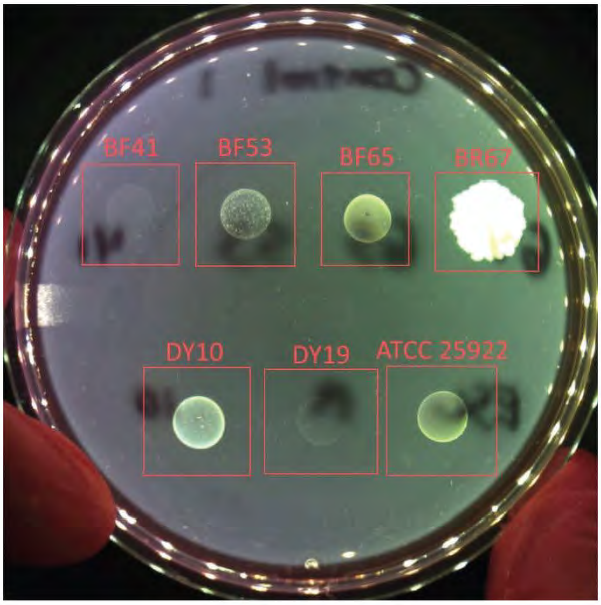
Table C-7. Agar Dilution Antibiotic Results and Susceptibility Photos: Streptomycin

	Streptomycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

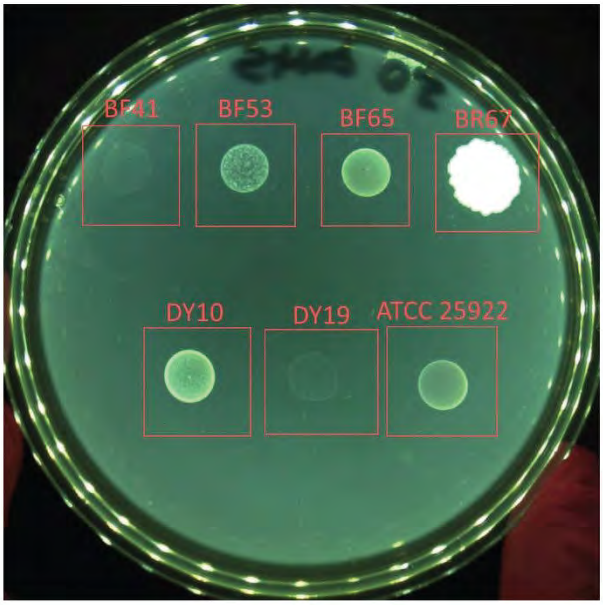
G = Growth

NG = No Growth

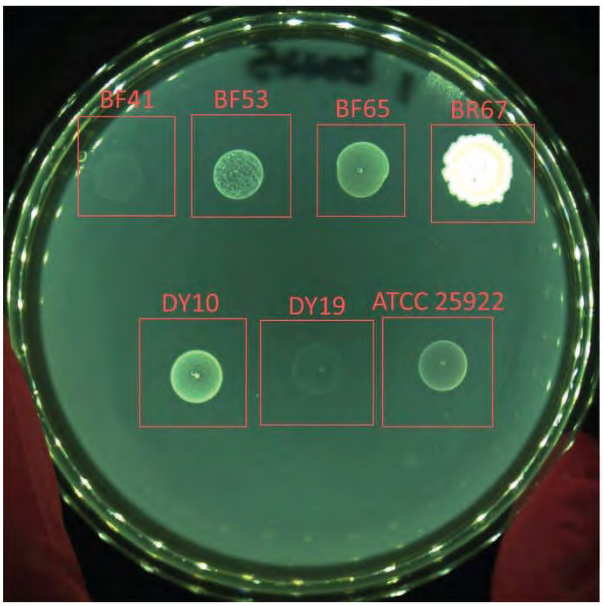
Agar Dilution Antibiotic Susceptibility Photos: Streptomycin



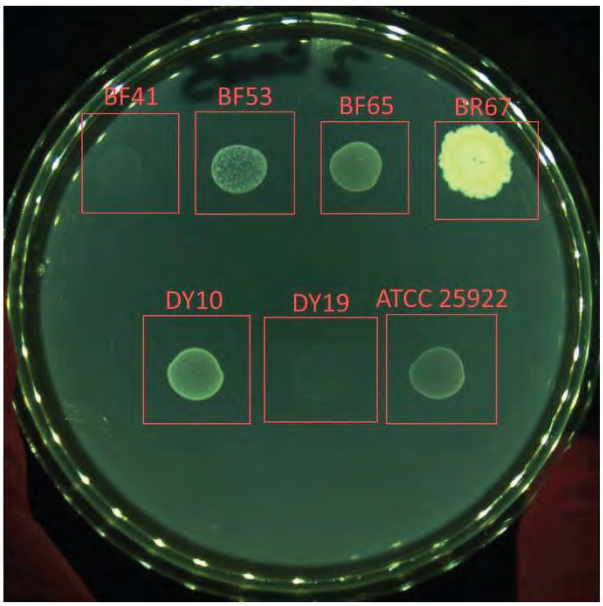
0 µg/mL Streptomycin



0.5 µg/mL Streptomycin



1 µg/mL Streptomycin



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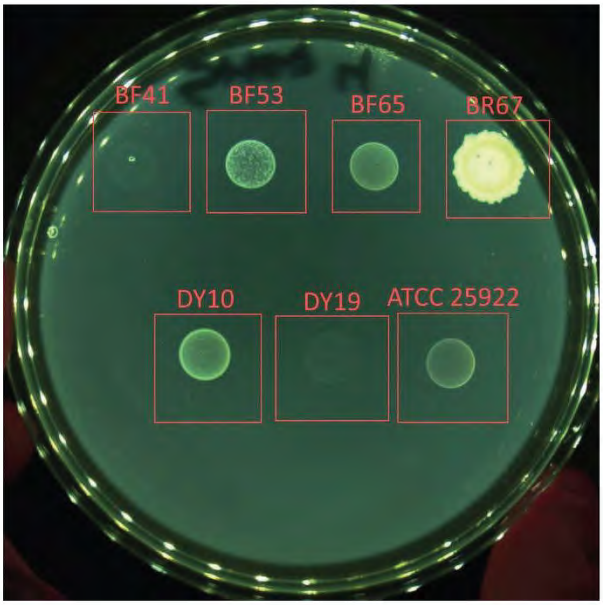
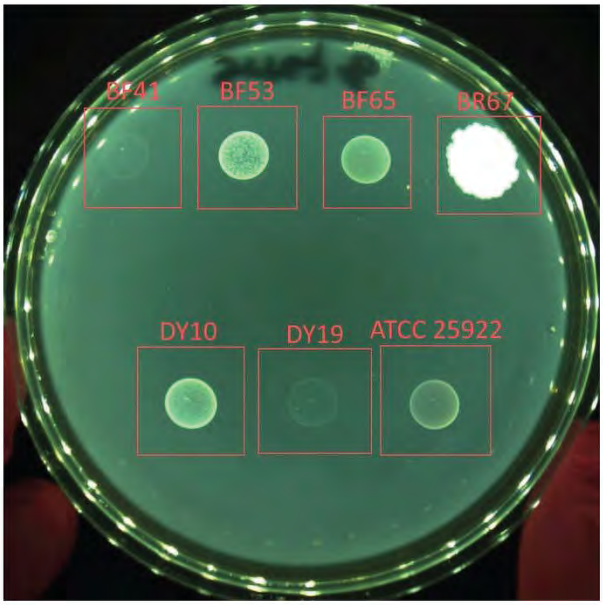
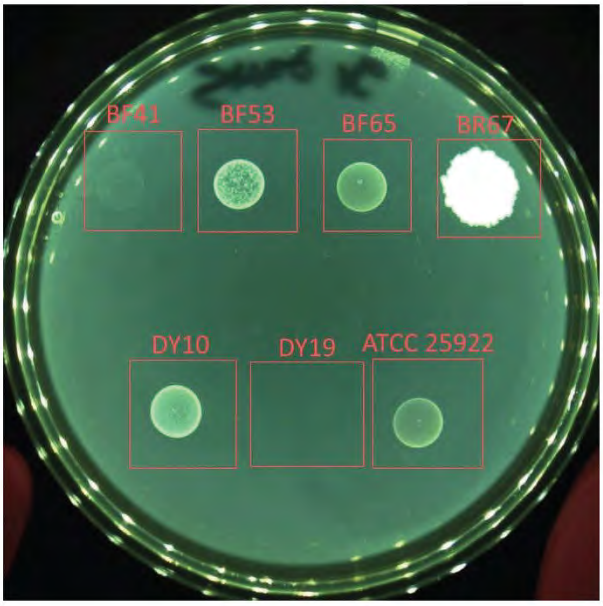


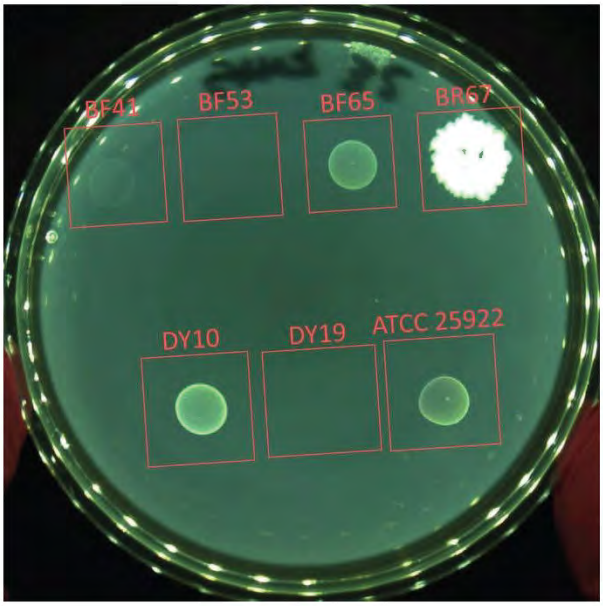
4 µg/mL Streptomycin



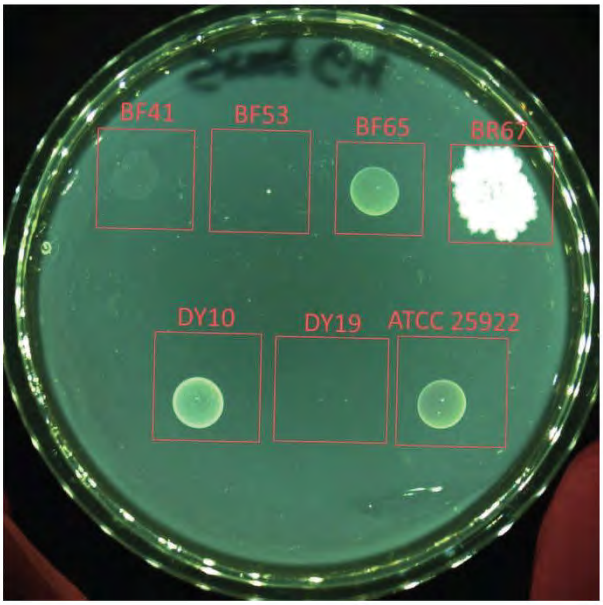
8 µg/mL Streptomycin



16 µg/mL Streptomycin



32 µg/mL Streptomycin



64 µg/mL Streptomycin

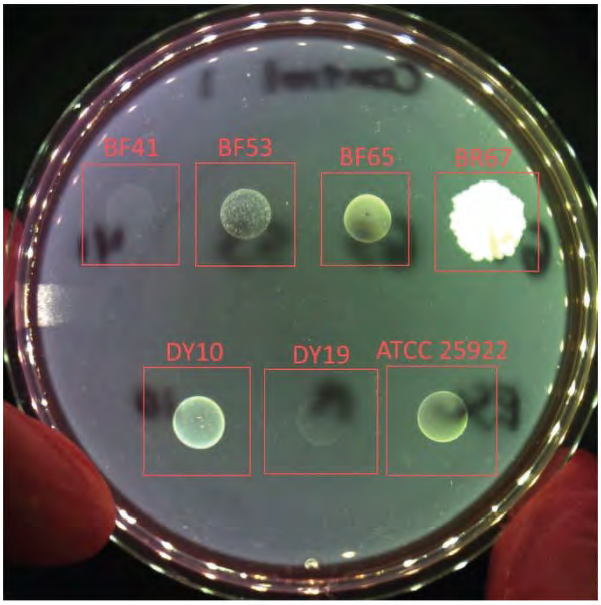
Table C-8. Agar Dilution Antibiotic Results and Susceptibility Photos: Tetracycline

	Tetracycline Concentration (µg/mL)											
Organism	**0 (Control)**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G	G	G	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	NG	NG	NG	NG	NG	NG

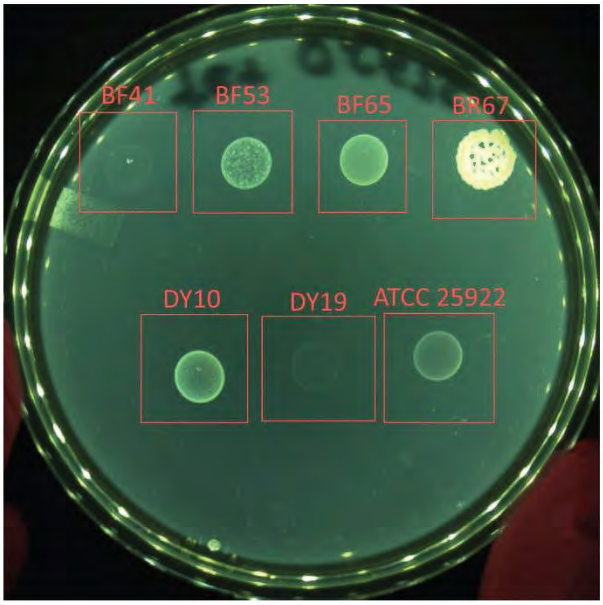
G = Growth

NG = No Growth

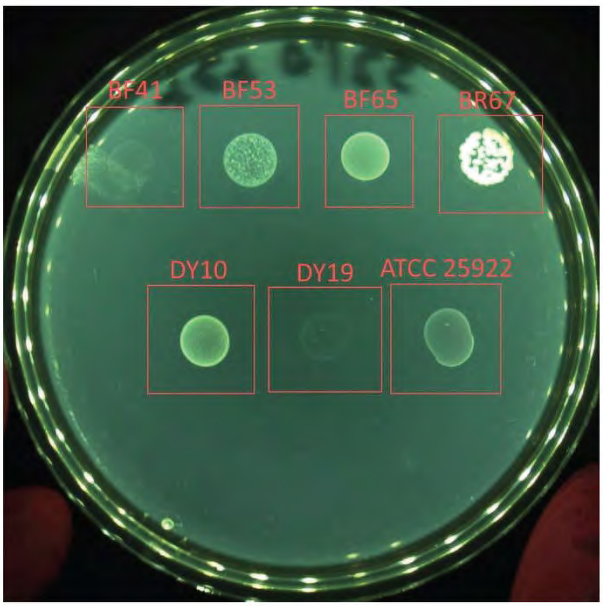
Agar Dilution Antibiotic Susceptibility Photos: Tetracycline



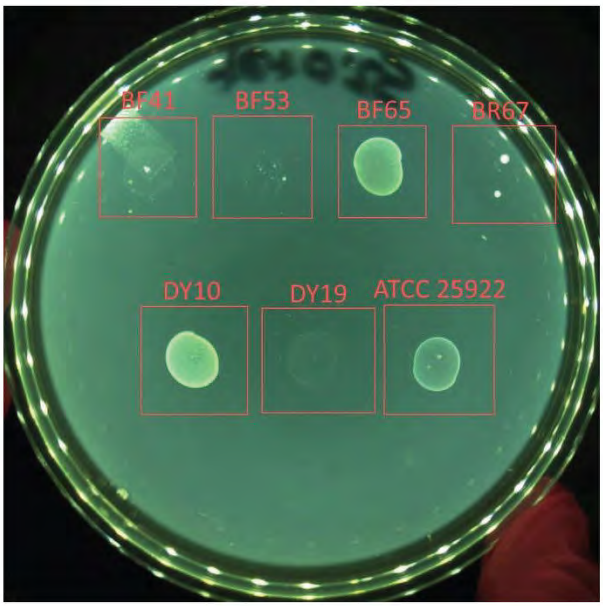
0 µg/mL Tetracycline



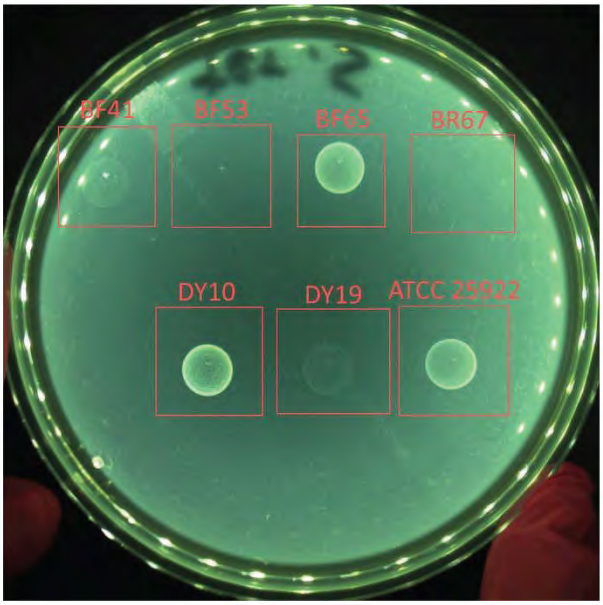
0.0625 µg/mL Tetracycline



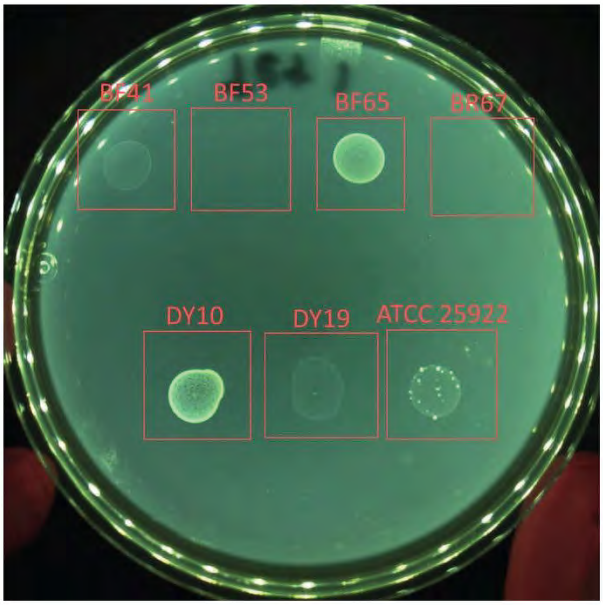
0.125 µg/mL Tetracycline



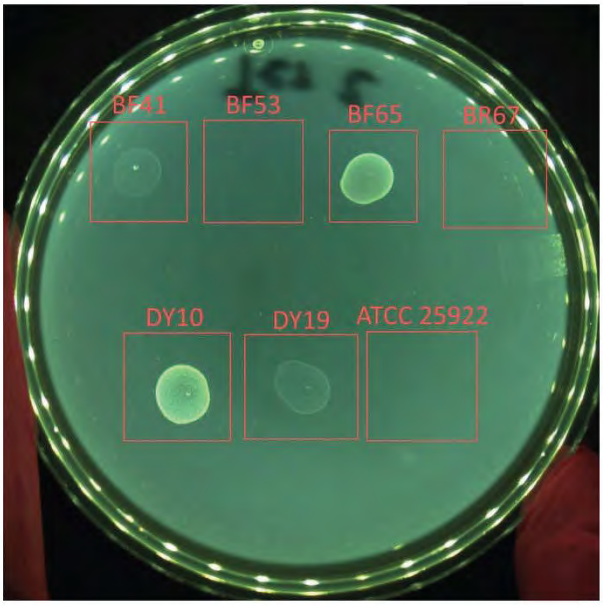
0.25 µg/mL Tetracycline



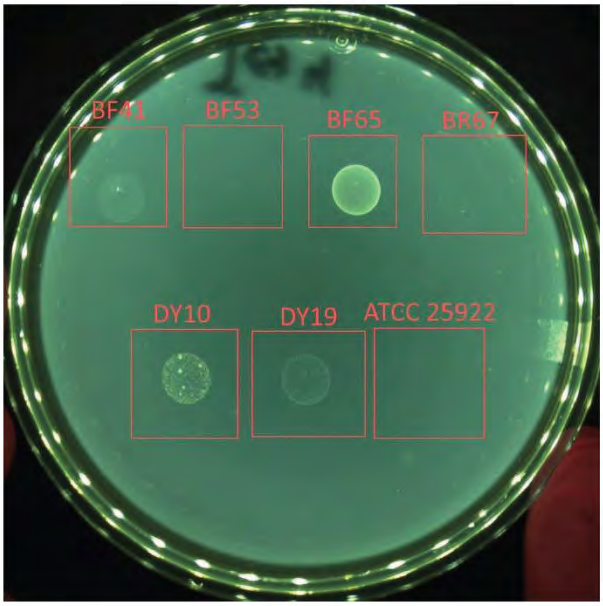
0.5 µg/mL Tetracycline



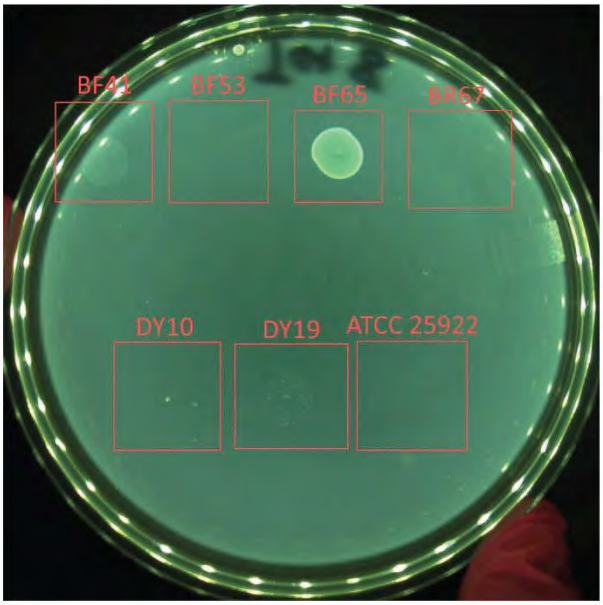
1 µg/mL Tetracycline



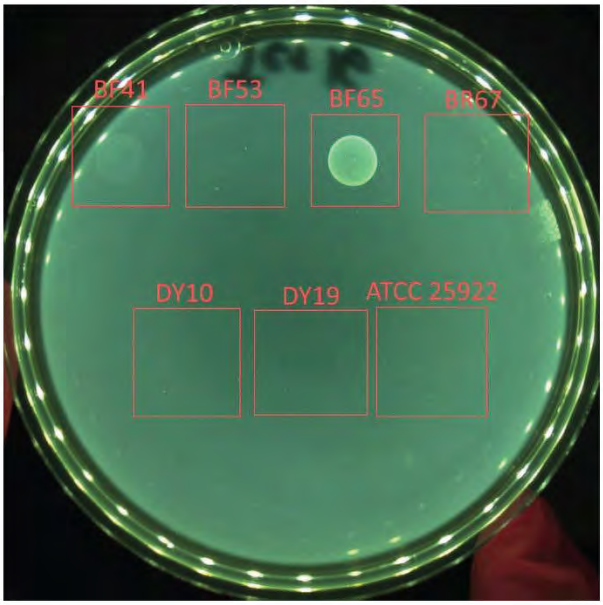
2 µg/mL Tetracycline



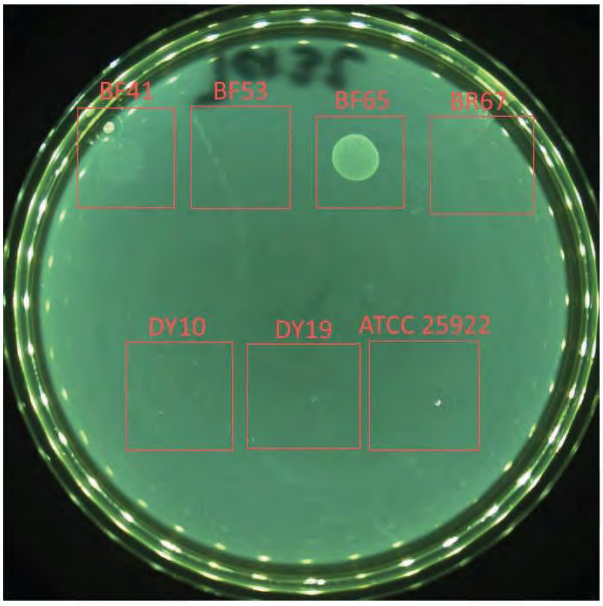
4 µg/mL Tetracycline



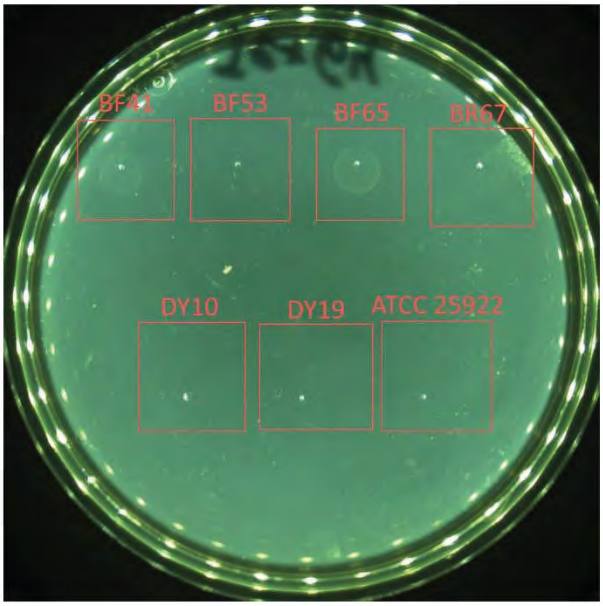
8 µg/mL Tetracycline



16 µg/mL Tetracycline



32 µg/mL Tetracycline



64 µg/mL Tetracycline

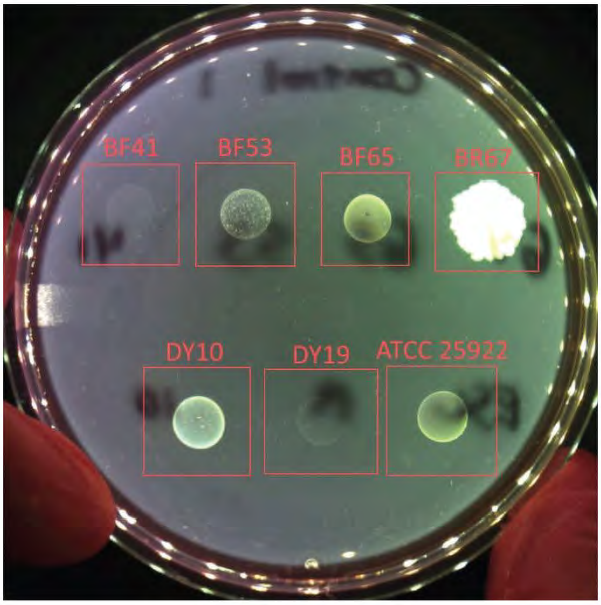
Table C-9. Agar Dilution Antibiotic Results and Susceptibility Photos: Vancomycin

	Vancomycin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	NG	NG
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	NG	NG	NG	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G

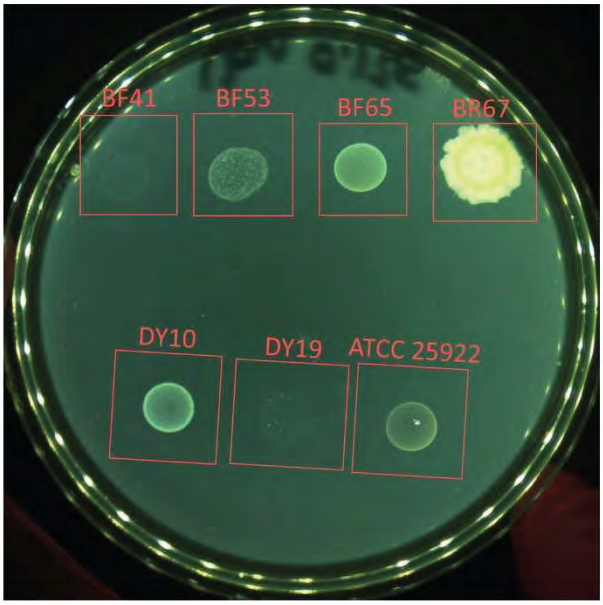
G = Growth

NG = No Growth

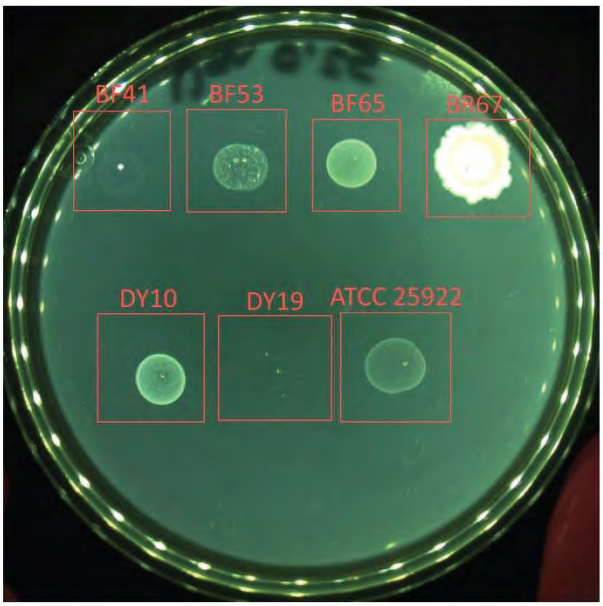
Agar Dilution Antibiotic Susceptibility Photos: Vancomycin



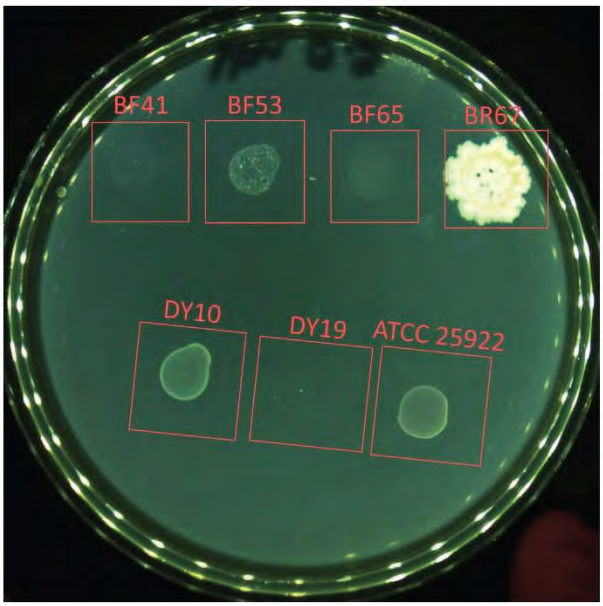
0 µg/mL Vancomycin



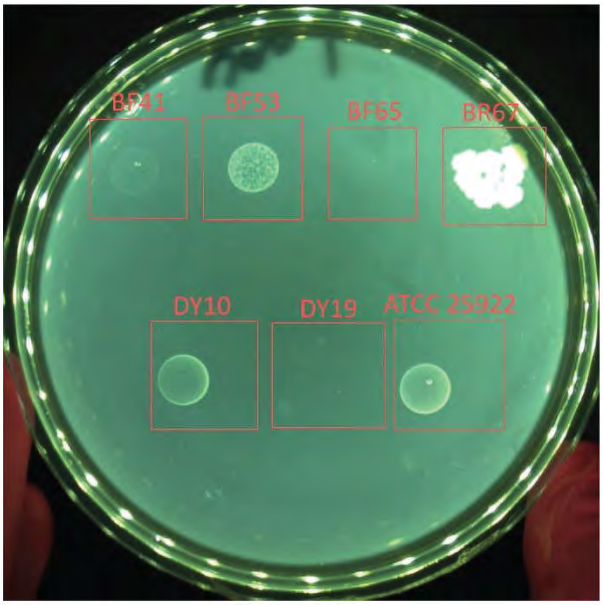
0.125 µg/mL Vancomycin



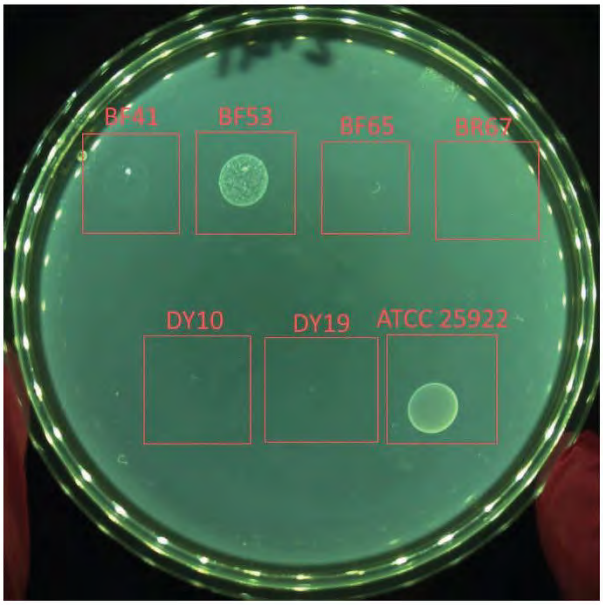
0.25 µg/mL Vancomycin



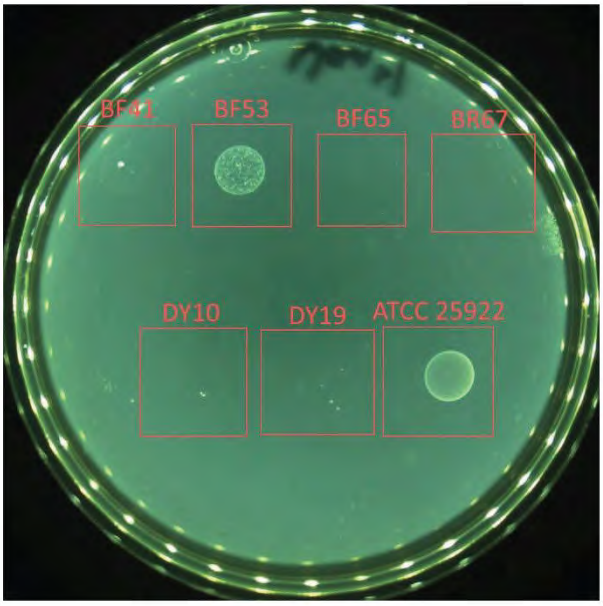
0.5 µg/mL Vancomycin



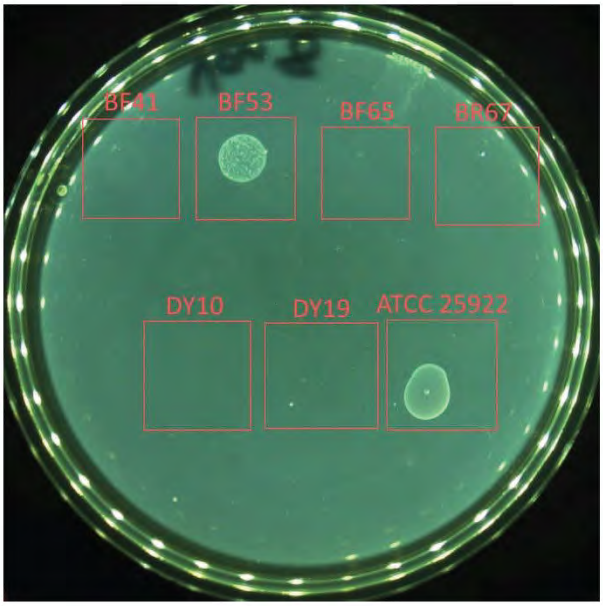
1 µg/mL Vancomycin



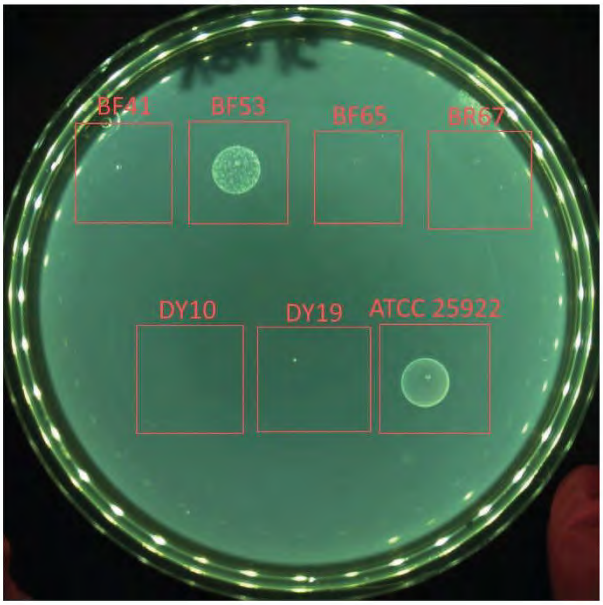
2 µg/mL Vancomycin



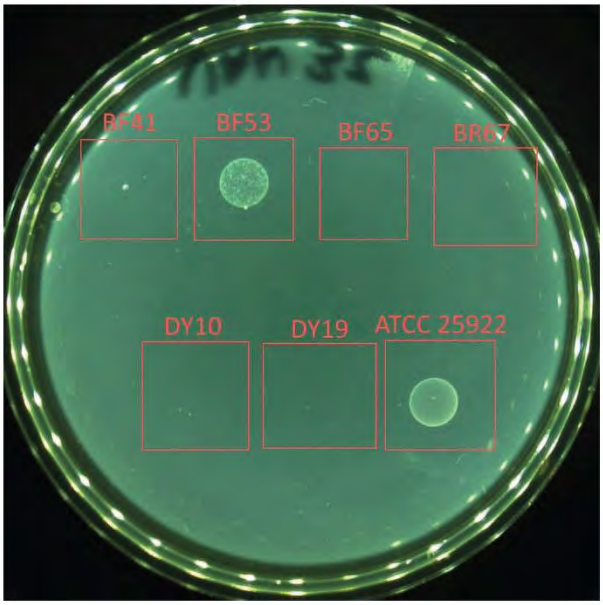
4 µg/mL Vancomycin



8 µg/mL Vancomycin



16 µg/mL Vancomycin



32 µg/mL Vancomycin

(b) (4)

FINAL REPORT

TITLE: **Characterization of Ascus Biosciences *Ruminococcus bovis* ASCUSDY10 (Dairy-10) Production Strain: Absence of Antimicrobial Activity**

INVESTIGATOR'S STUDY NUMBER: (b) (4)

CONDUCT DATES: Receipt of supernatant: November 20, 2019
Testing of supernatant: November 27, 2019 – December 5, 2019

SPONSOR: Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121

INVESTIGATOR: (b) (4)

VERSION: FINAL

SIGNATURE: (b) (6) _____ 2/21/20
Principal Investigator Date

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LIST OF TABLES AND APPENDICES

OBJECTIVES

To determine the antimicrobial properties of the *Ruminococcus bovis* ASCUSDY10 (Dairy-10) production strain supernatant.

STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in the protocol.

STUDY SITE

Antimicrobial property testing of the product was performed by (b) (4)

MATERIALS

The sponsor provided Dairy-10 supernatant (Lot number (b) (4)) was prepared by (b) (4) followed by sterile filtration with a (b) (4) membrane. The sample was received on November 20, 2019.

ANTIMICROBIAL PROPERTIES

A portion of the growth medium from a typical production batch of the *Ruminococcus bovis* ASCUSDY10 (Dairy-10), or a scaled down version, was kept refrigerated (2-8°C) and shipped to (b) (4) and used 13 days after receipt.

1.1. Preparation of Culture Plates

The following six organisms were tested against the supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	(b) (4)	1:10
Escherichia coli	11229		1:10
Bacillus cereus	2		1:10
Bacillus circulans	4516		1:10
Streptococcus pyogenes	12344		1:20
Serratia marcescens	14041		1:10

(b) (4)

1.2. Disk Preparation

(b) (4)

1.3. Incubation

(b) (4)

1.4. Interpretation

(b) (4)

1.5. Quality Control

(b) (4)

DISPOSITIONS

The supernatant was discarded after autoclaving and issue of the final report. No retention sample was maintained.

RESULTS

No zones of inhibition were observed for the Dairy-10 supernatant lot, or the sterile distilled water control. A zone of inhibition was observed for the enrofloxacin positive control for each organism as indicated in the table below:

Table 1. Zone Diameters from Dairy-10 Supernatant and Controls

Organism	ATCC number	(b) (4) code	Zone Diameter for the indicated solution (mm)		
			Dairy-10 Supernatant	Sterile Distilled water	Enrofloxacin
Staphylococcus aureus	6538	(b) (4)			
Escherichia coli	11229				
Bacillus cereus	2				
Bacillus circulans	4516				
Streptococcus pyogenes	12344				
Serratia marcescens	14041				

(b) (4)

Following incubation, pictures were taken of each organism seeded into the agar onto which a saturated disk of supernatant and controls were placed according to the protocol. These pictures are included in Appendix B. No zones of inhibition are observed in these pictures.

CONCLUSION

The Dairy-10 supernatant exhibited no antibacterial activity against the 6 strains representative of Gram positive and Gram negative bacteria.

APPENDIX A. Protocol

(b) (4)

STUDY PROTOCOL

TITLE:	**Characterization of Ascus Biosciences Various Production Strain: Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (6), (b) (4)
VERSION:	FINAL

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SIGNATURES

Sponsor Representative

Jordan Embree
Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121
Email: jordan@ascusbiosciences.com
Tel. 877-696-8945 x709

(b) (6) 11/14/19
Date

Investigator

(b) (6), (b) (4)

(b) (6) 11/14/19
Signature Date

1. OBJECTIVES

Determination of the antimicrobial properties of various production strain supernatants.

2. STUDY TIMELINE

Anticipated study dates are:
Antimicrobial Properties: November 2019

3. STANDARDS OF COMPLIANCE

This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in this protocol.

4. STUDY SITE

Antimicrobial properties testing of the products will be performed by (b) (4) (b) (4)

5. MATERIALS AND METHODS

5.1. Supernatant

It is anticipated that 5 supernatants will be provided by the Sponsor. The supernatants will be streaked onto trypticase soy agar containing (b) (4) (b) (4)

6. ABSENCE OF ANTIMICROBIAL PRODUCTION[1]

The presence of antimicrobial activity in the growth medium from the production strain supernatants will be tested. (b) (4) (b) (4)

[1] FAO (2006) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122.

6.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant: Volumes of media and numbers of plates should be adjusted as required, based upon the number of supernatants tested.

Organism	ATCC number	(b) (4)	Dilution tested
Staphylococcus aureus	6538	(b) (4)	1:10
Escherichia coli	11229		1:10
Bacillus cereus	2		1:10
Bacillus circulans	4516		1:10
Streptococcus pyogenes	12344		1:20
Serratia marcescens	14041		1:10

(b) (4)

6.2. Disk Preparation

(b) (4)

6.3. Incubation

(b) (4)

(b) (4)

6.4. Interpretation

(b) (4)

6.5. Quality Control

(b) (4)

7. RAW DATA, RECORDS, AND REPORTS

7.1. Data

All raw data will be recorded, handled, and stored according to facility SOPs, this protocol, and applicable regulatory requirements. All original data collected and records generated in connection with the study will be archived at the study site. The following records will be maintained:

- Quality control records generated concurrent with all media and materials preparation, and lab testing.
- Protocols, protocol amendments, correspondence, reports and other documentation, including drafts of the final report
- Raw data and logs
- Documents related to any occurrence or situation that develops during the course of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is ongoing, and thereafter in archives storage at (b) (4)

7.2. Reporting of Results

A separate report will be issued for the production strain for each of the tests performed. If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

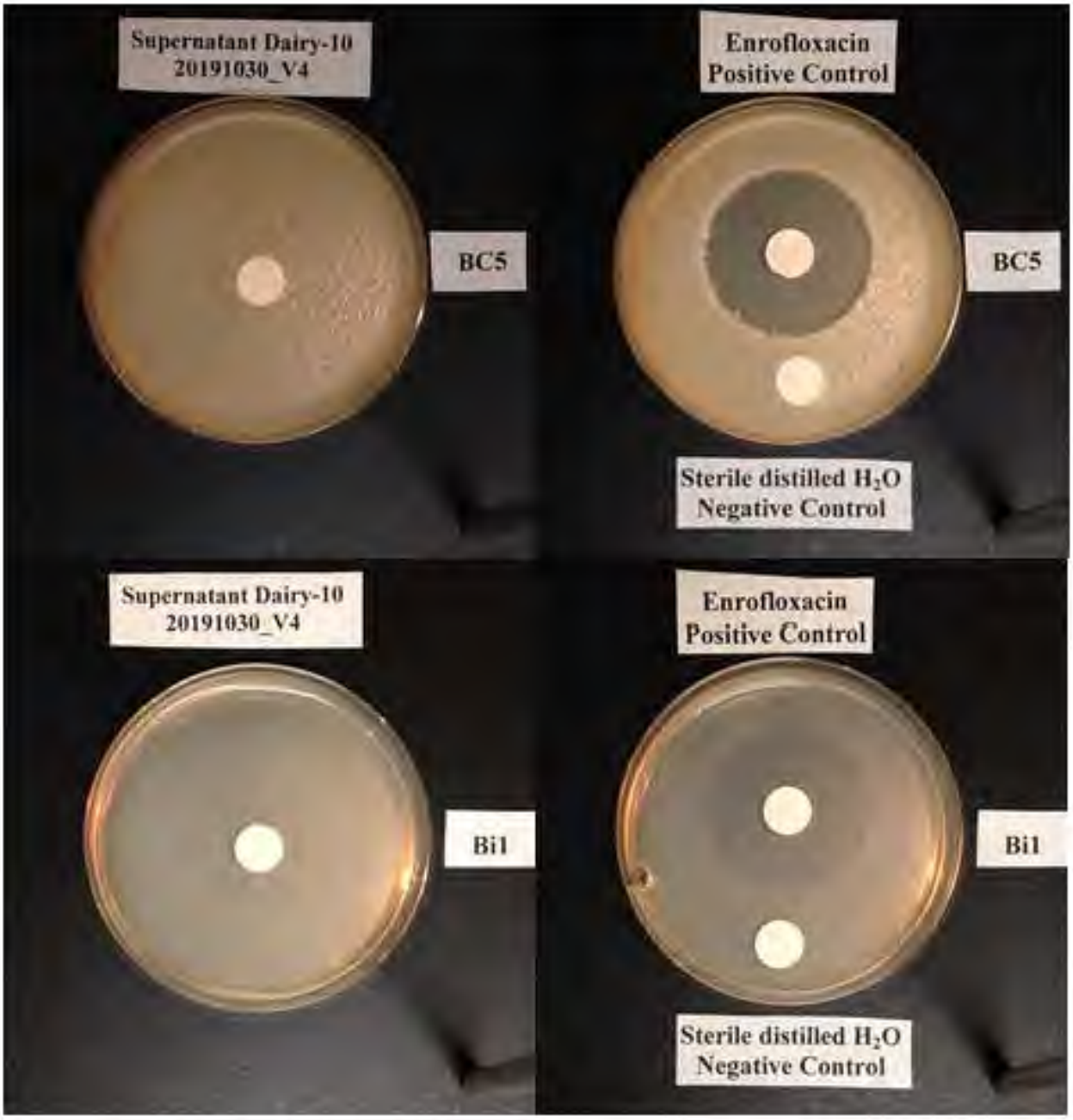
8. DISPOSITIONS

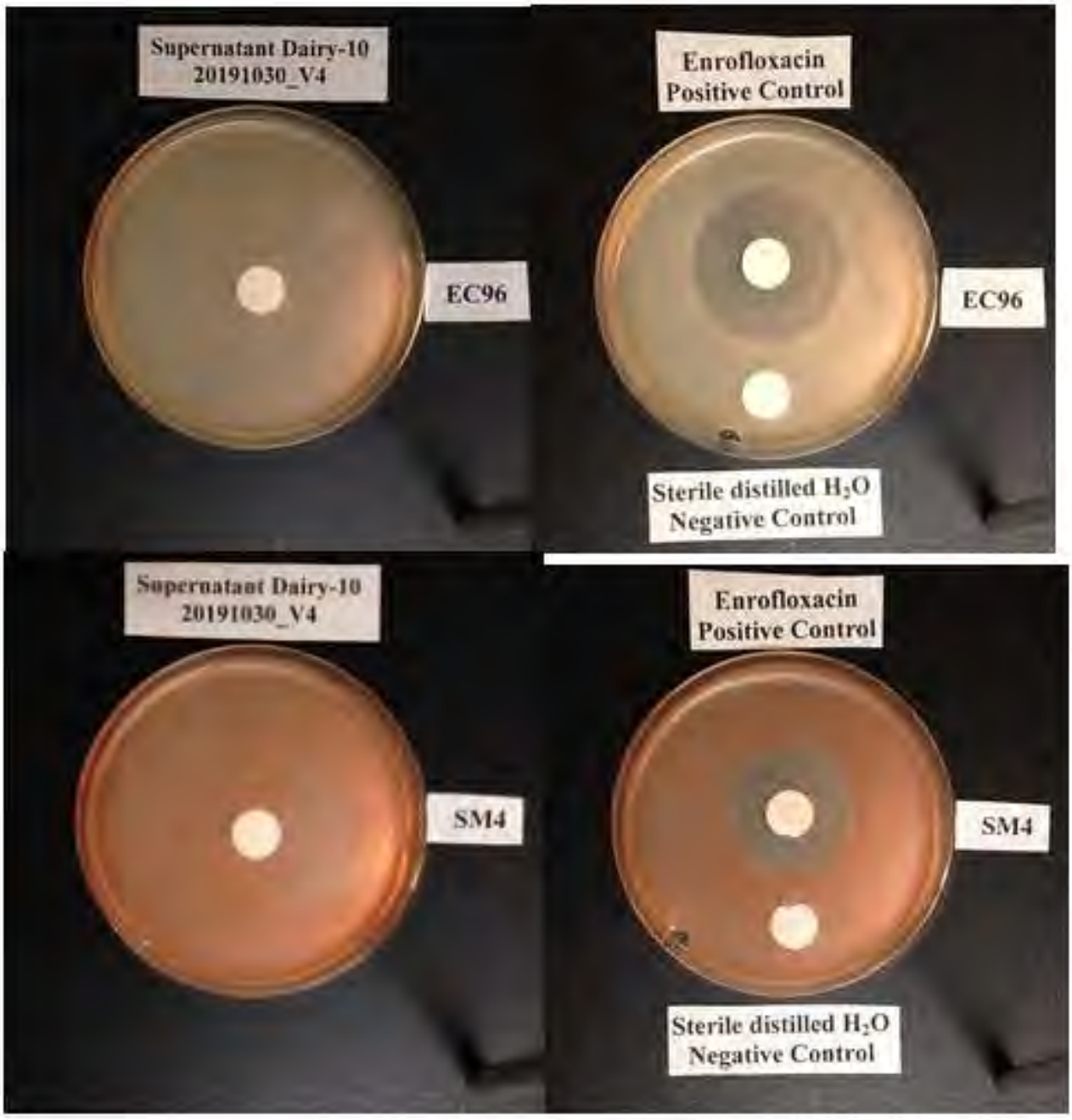
8.1. Supernatants
All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

9. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment that will be maintained with the protocol. As a minimum, the amendment will indicate the changes or revisions made, indicate the effective date, identify the protocol sections affected, explain the reasons for change and describe the impact on the study. The amendment will be signed and dated by those who signed the protocol. Signatures will be obtained before implementation of the change if possible. If such is not possible, the investigator will attempt to obtain verbal prior authorization from the sponsor and follow with written documentation at the earliest opportunity. Protocol deviations are defined as unintended or unforeseeable necessary changes to the protocol. Protocol deviation reports list any action that is not/was not in accordance with the protocol. They must contain a detailed description of the deviation, its reason, and a description of its effect on the study.

APPENDIX B: Photos

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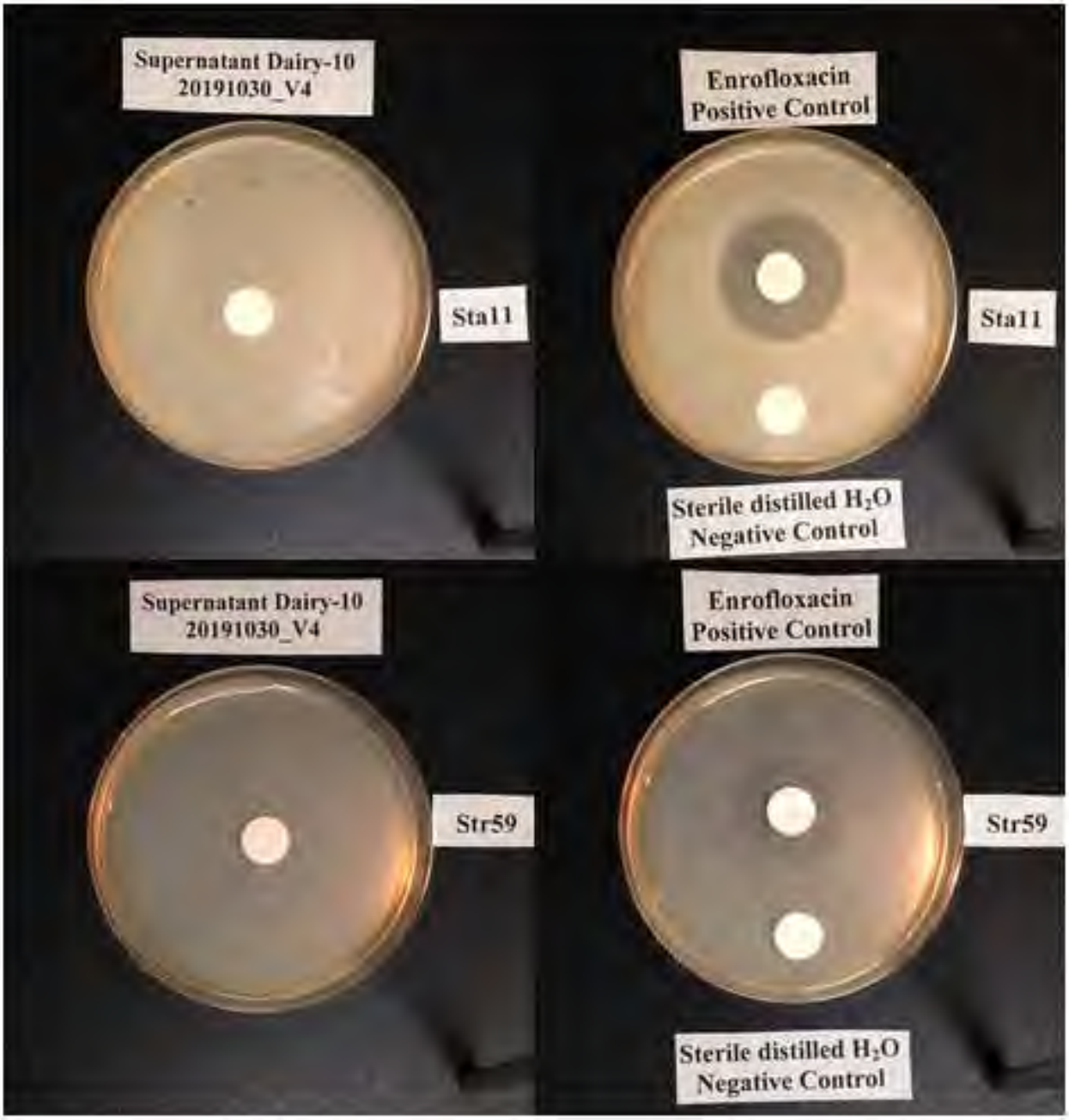
20191030_V4
EC96
Enrofloxacin
Positive Control
EC96
Sterile distilled H_2O
Negative Control
Supernatant Dairy-10
20191030_V4
SM4
Enrofloxacin
Positive Control
SM4
Sterile distilled H_2O
Negative Control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Sta11
Enrofloxacin
Positive Control
Sta11
Sterile distilled H_2O
Negative Control
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Str59
Enrofloxacin
Positive Control
Str59
Sterile distilled H_2O
Negative Control

Appendix 6 withheld entirely

Confidential Information under FOIA Exemption (b)(4)

OXFORD

Journal of AOAC INTERNATIONAL, 103(2), 2020, 513–522

doi: 10.1093/jaocint/qsz013
Advance Access Publication Date: 12 March 2020
Article

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the 3M™ Petrifilm™ Rapid *E. coli*/Coliform Count Plate for the Enumeration of *E. coli* and Coliforms: Collaborative Study, First Action: 2018.13

(b) (4)

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Collaborators: A. Calle, K. Suntharesan, V. Gohil, A. Donkers, R. Smith, D. Wood, S. Diederich, S. Kuchenberg, I. Satoshi, M. Brown, N. Alvarez, S. Corti, M. Hochreuter
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Abstract

Background: The 3M™ Petrifilm™ Rapid *E. coli*/Coliform Count Plate is a selective and differential sample-ready-culture medium designed for the rapid enumeration of *Escherichia coli* (*E. coli*) and coliforms in the food and beverage industries.
Objective: The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was compared to the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) Chapter 4 Enumeration of *Escherichia coli* and the Coliform Bacteria, the International Organization of Standards (ISO) 4832:2006 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms—Colony-count technique, and ISO 16649-2:2017 Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli—Part 2 Colony-count technique at 44 degrees C using bromo-4-chloro-3- indolyl beta-D-glucuronide methods for the enumeration of *E. coli* and coliforms in dry dog kibble.
Method: The candidate method was evaluated using two diluents, Butterfield's phosphate buffered diluent and peptone salt solution, in a paired study design with each reference method in a multi-laboratory collaborative study following the current AOAC Validation Guidelines. Three target contamination levels and an uninoculated control level were evaluated.
Results: The candidate and reference methods were not statistically different at each contamination level. Reproducibility values obtained during the collaborative study were similar between the candidate and reference methods.
Conclusion: These results demonstrate that the candidate method is equivalent to the reference methods.
Highlight: 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was recommended for Official First Action status for enumeration of *E. coli* and coliforms in a broad range of foods and environmental surfaces.

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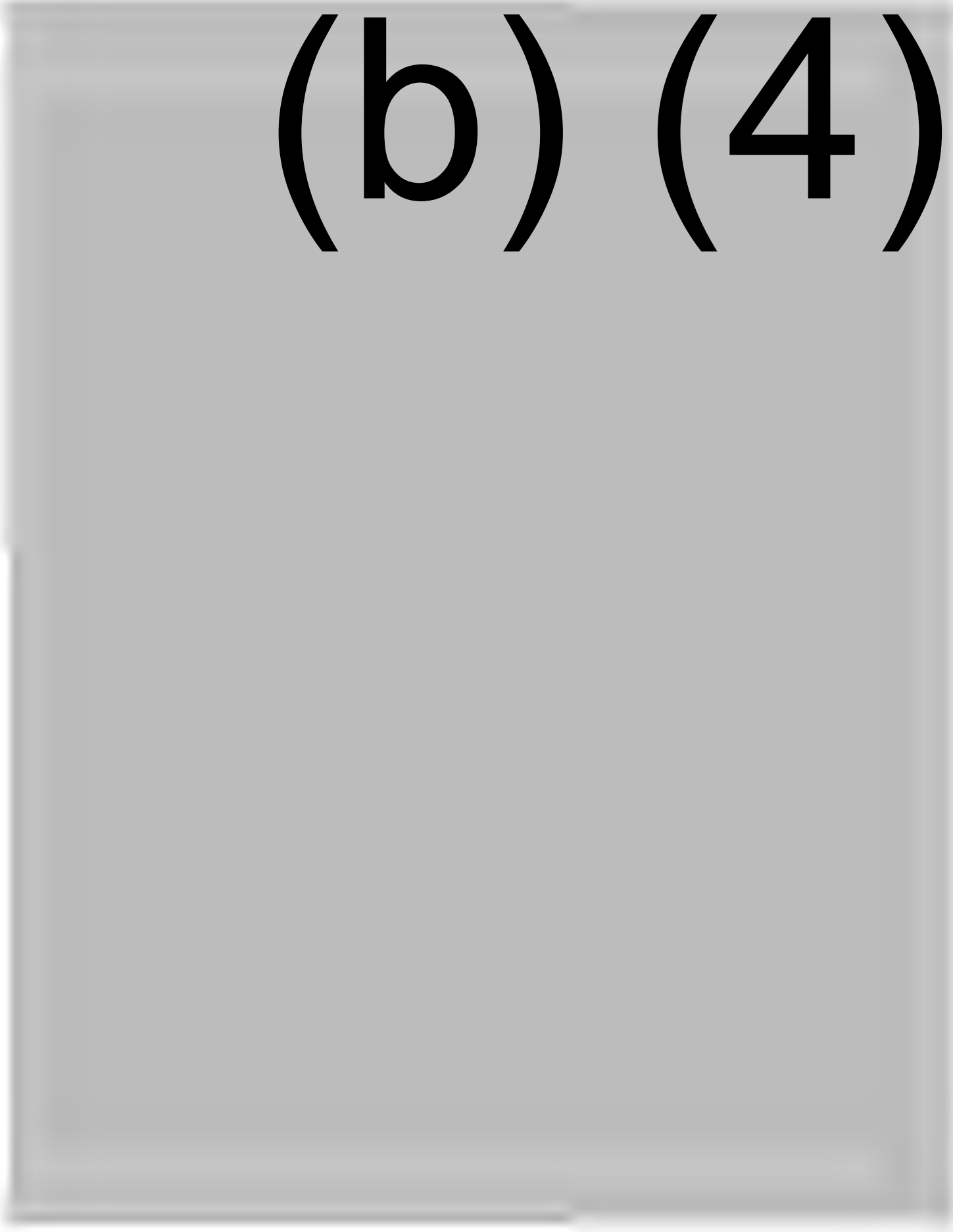
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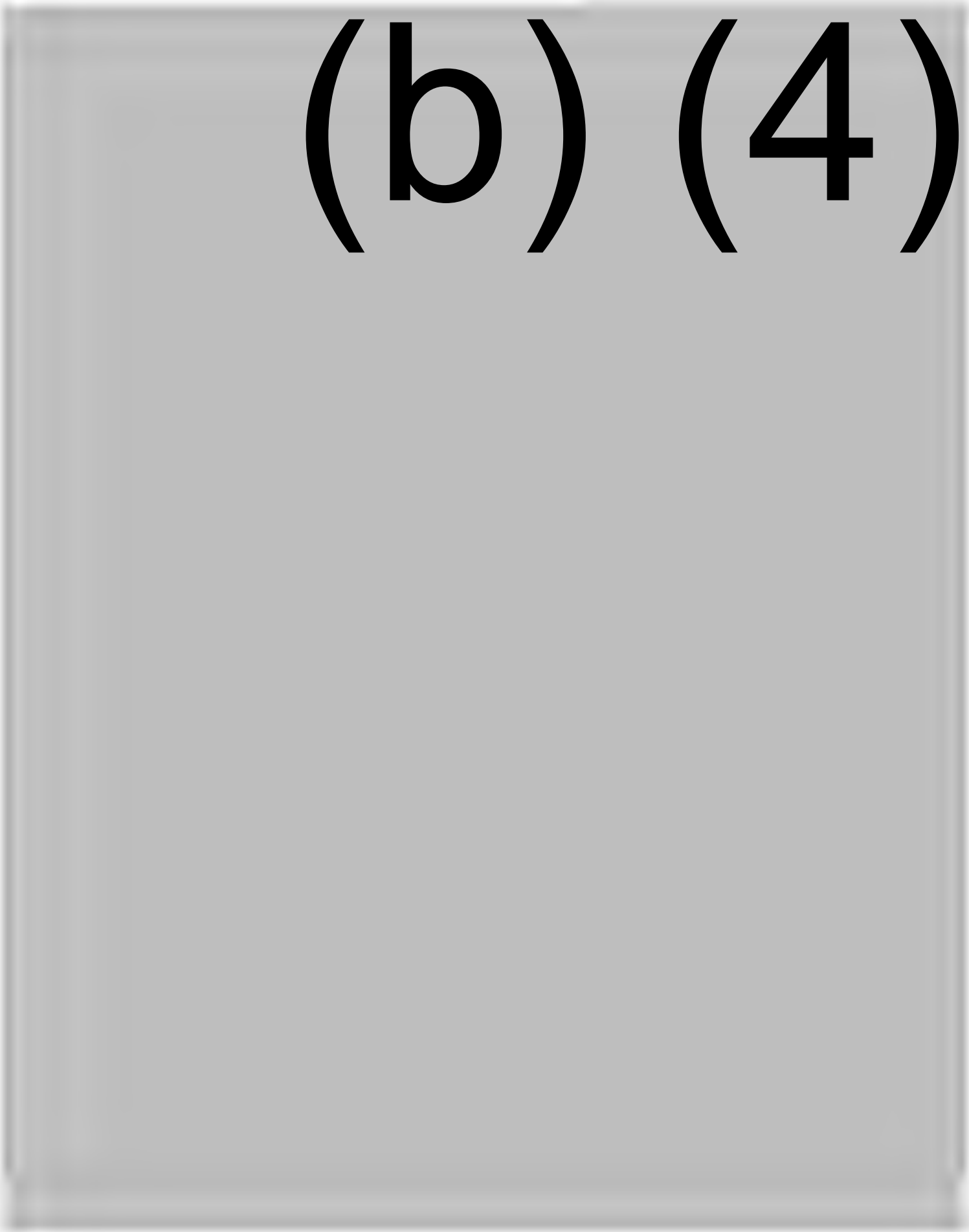
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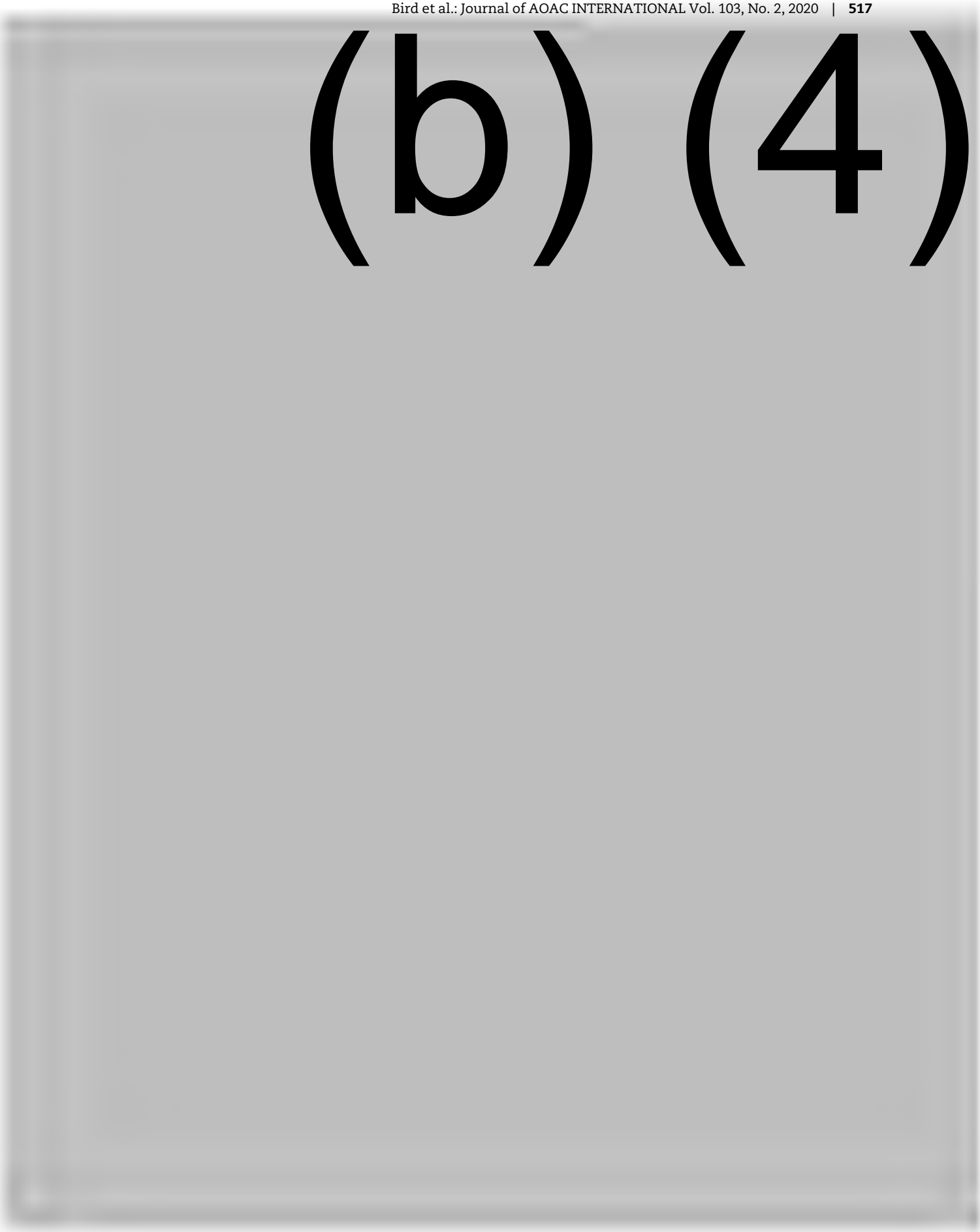
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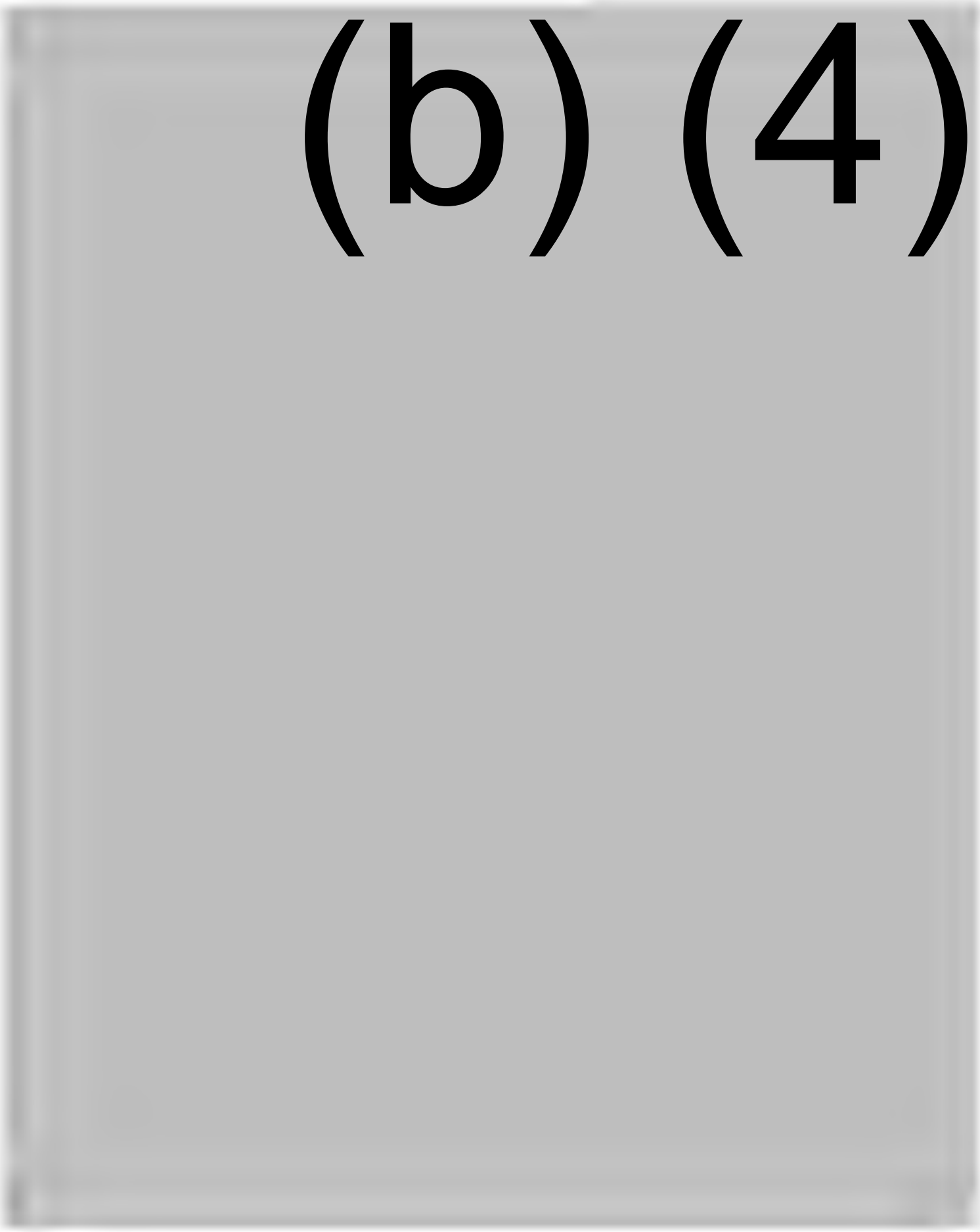
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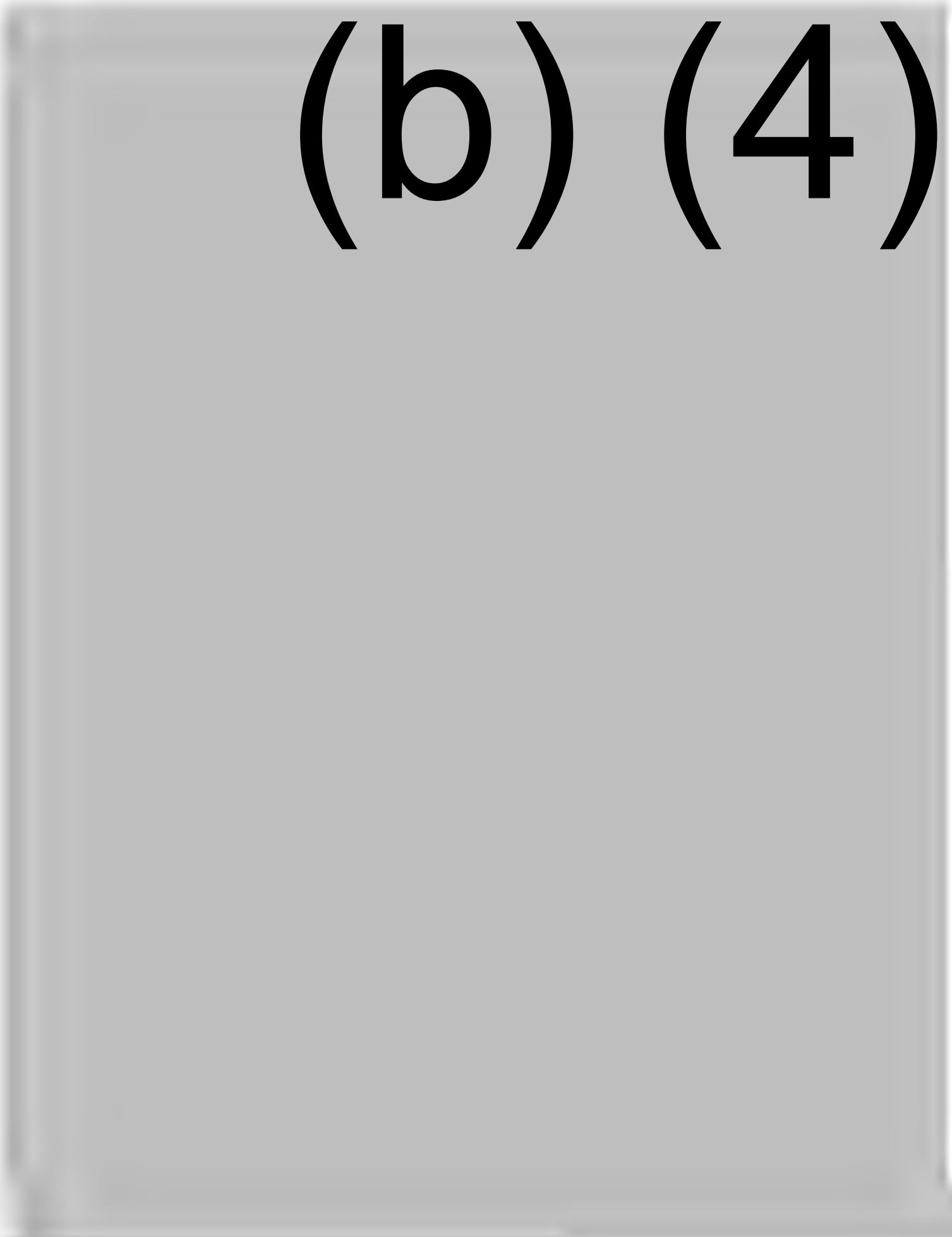
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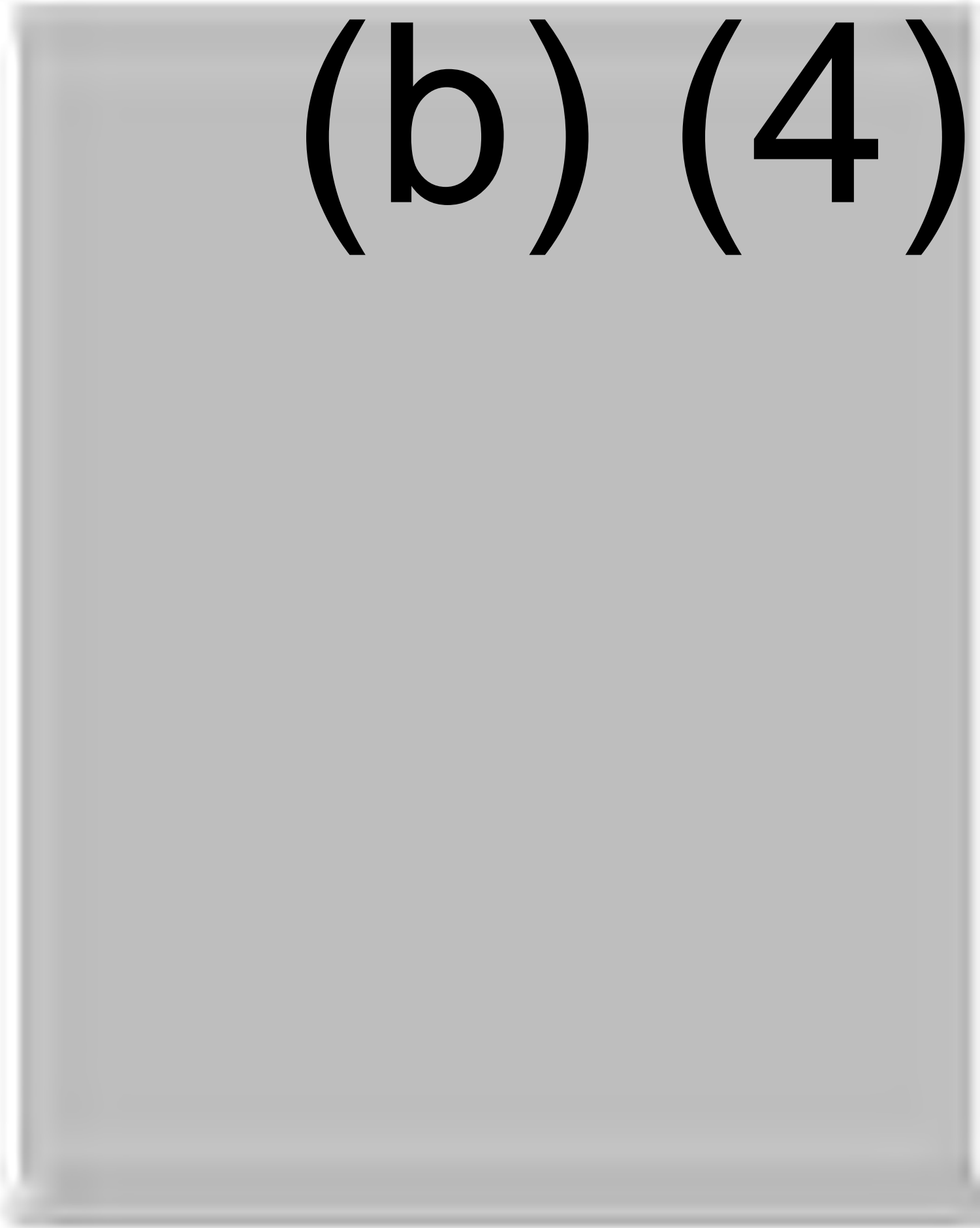
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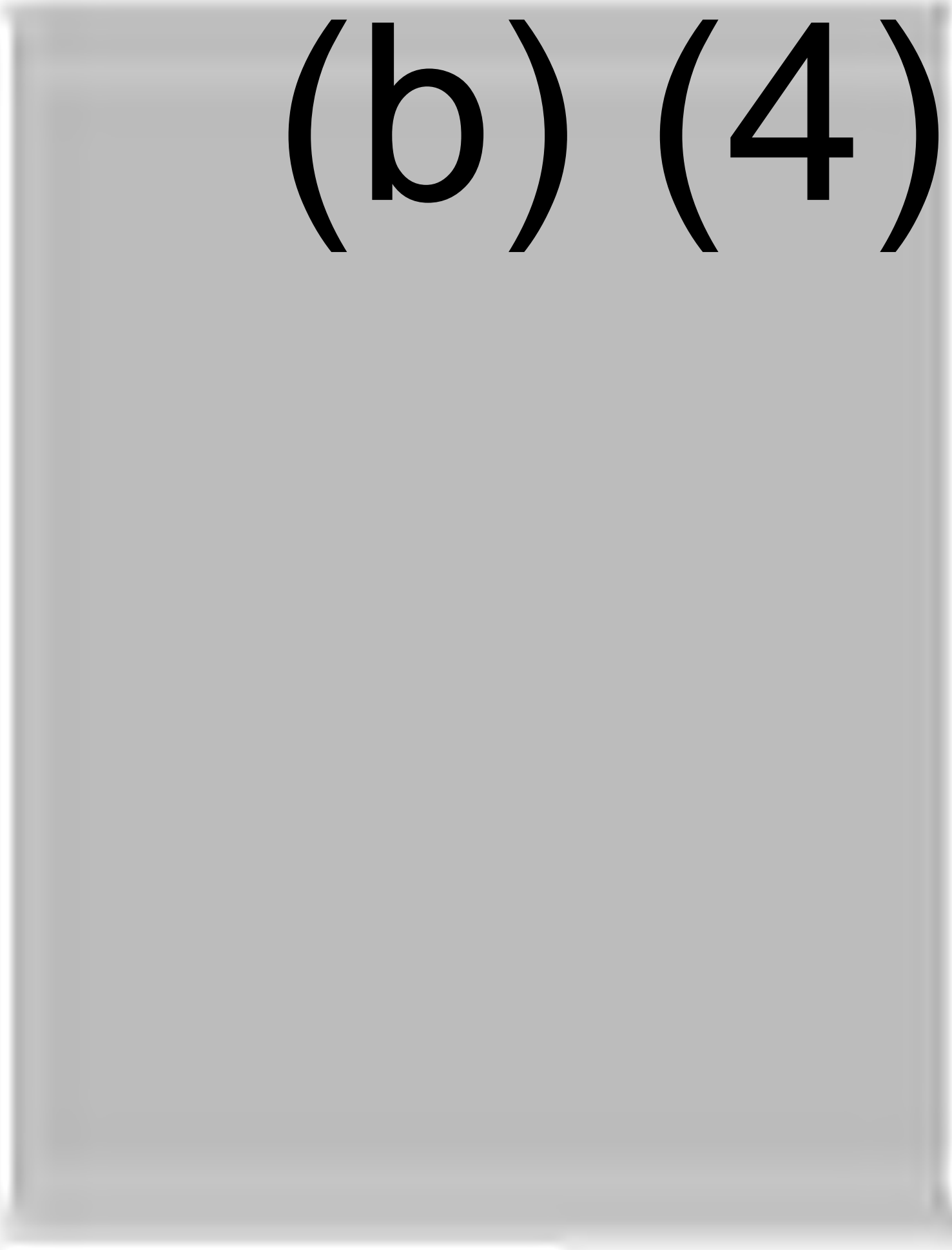
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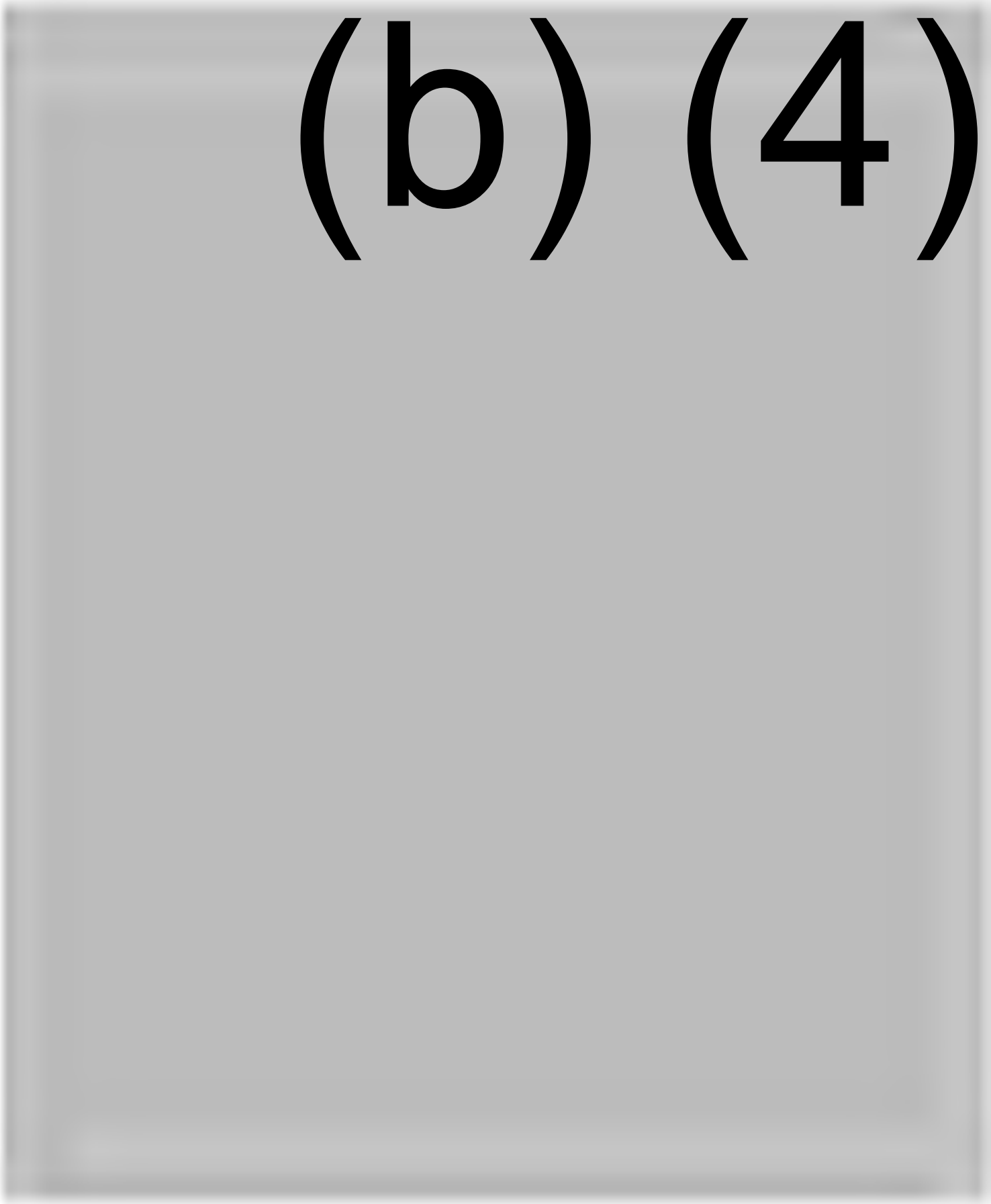
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BAM: Clostridium botulinum

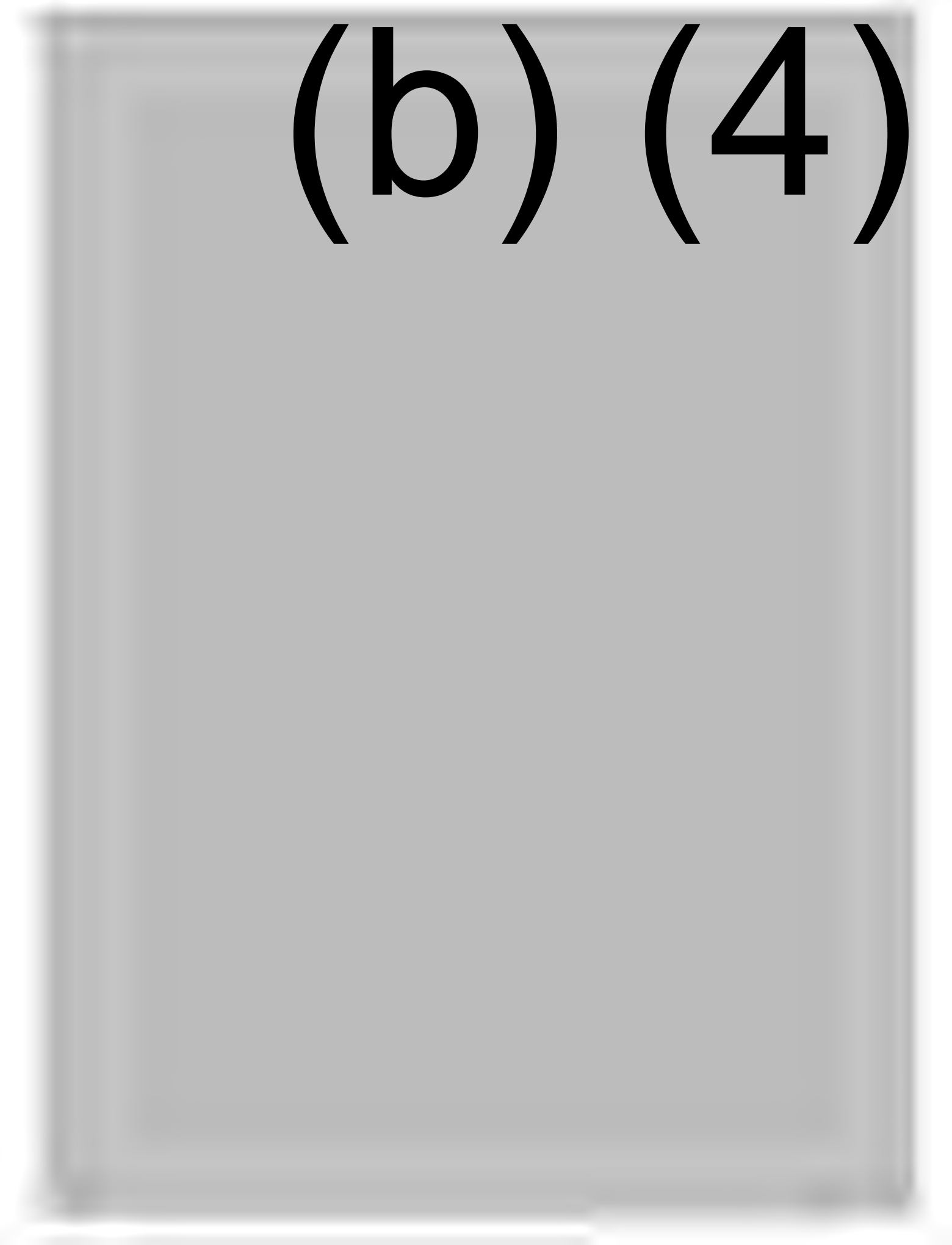
January 2001

Bacteriological Analytical Manual
Chapter 17
Clostridium botulinum

Authors: Haim M. Solomon and Timothy Lilly, Jr.

For additional information, contact Shashi Sharma
(mailto:Shashi.Sharma@fda.hhs.gov)

Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 (2) involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism.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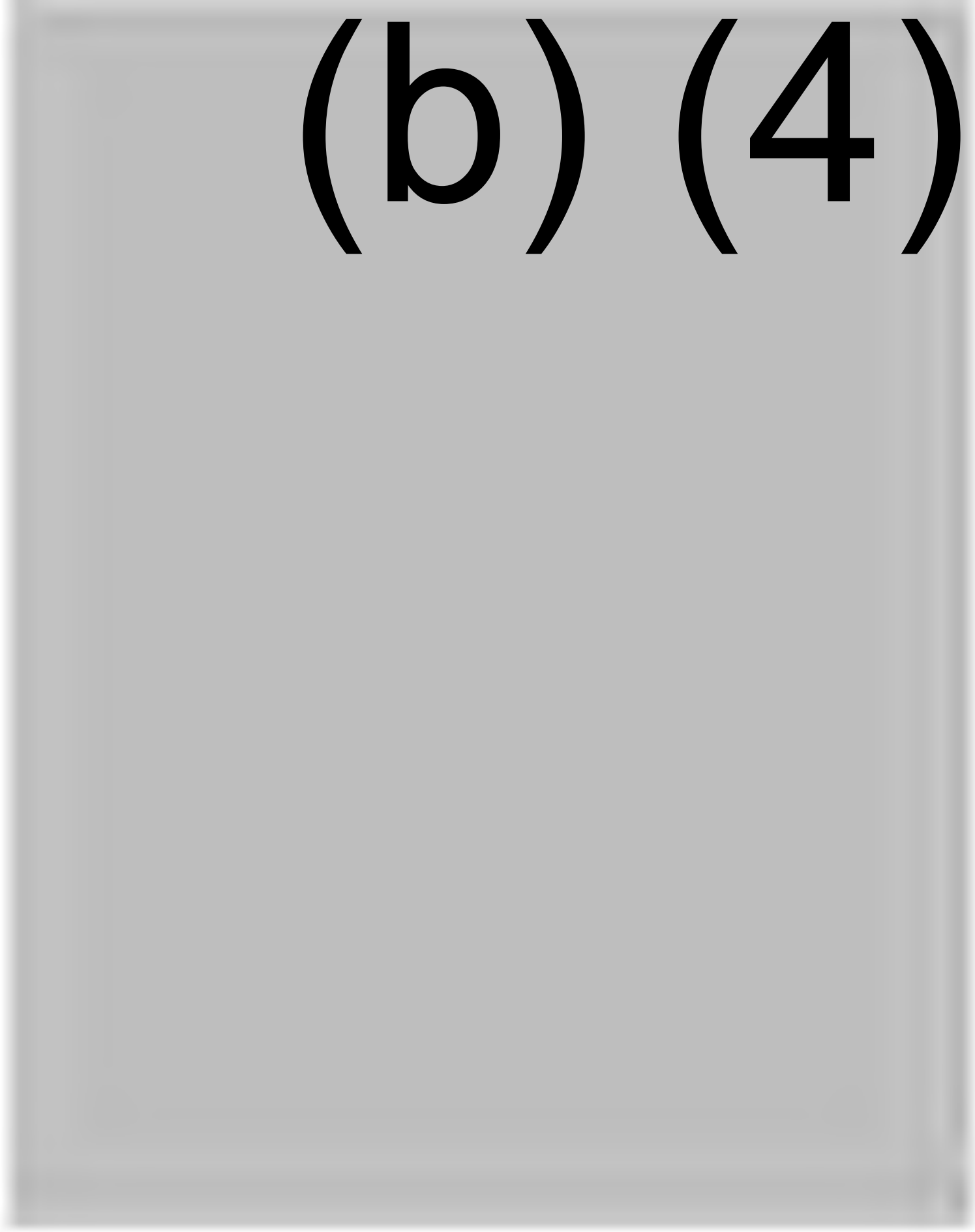
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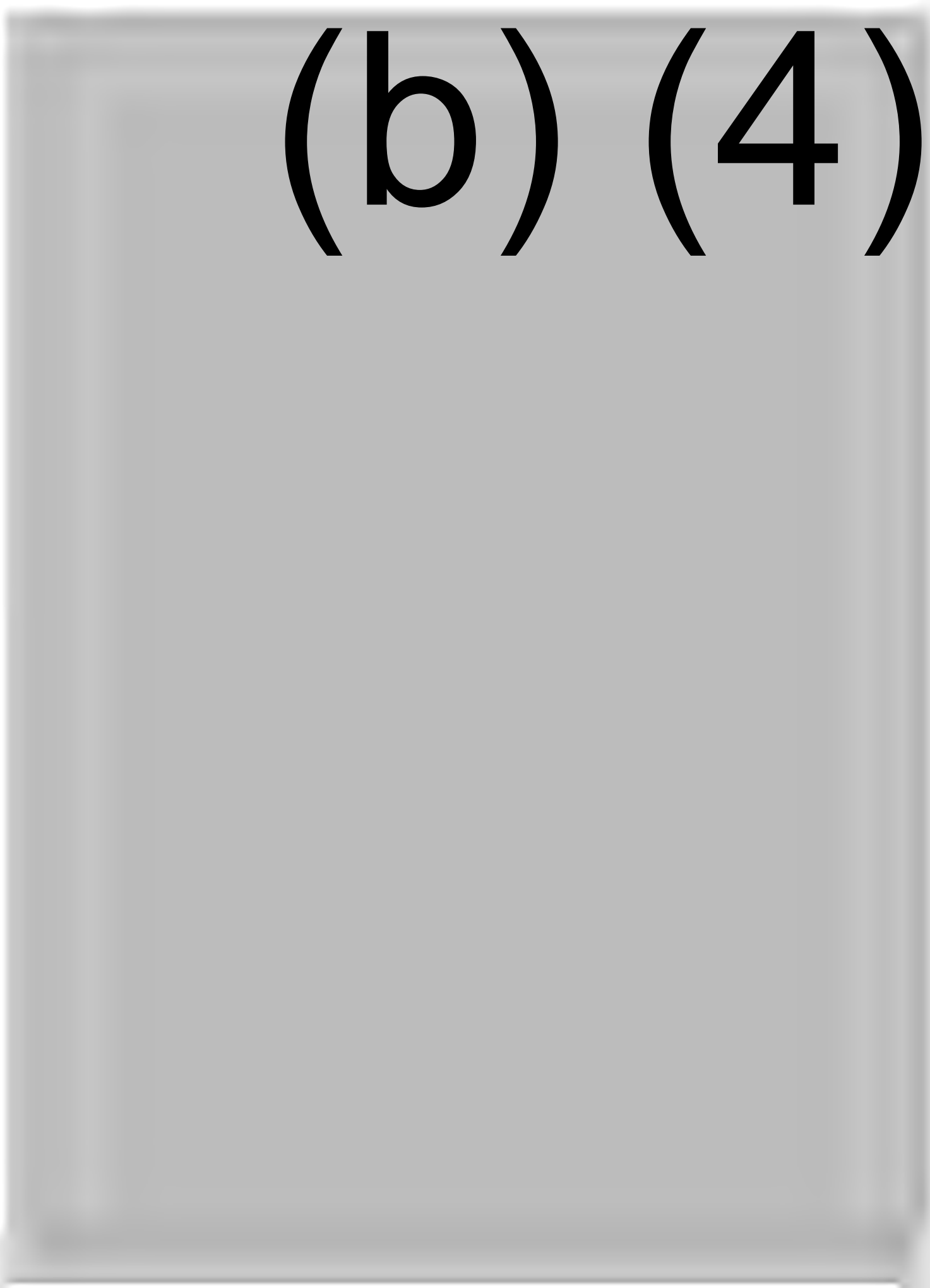
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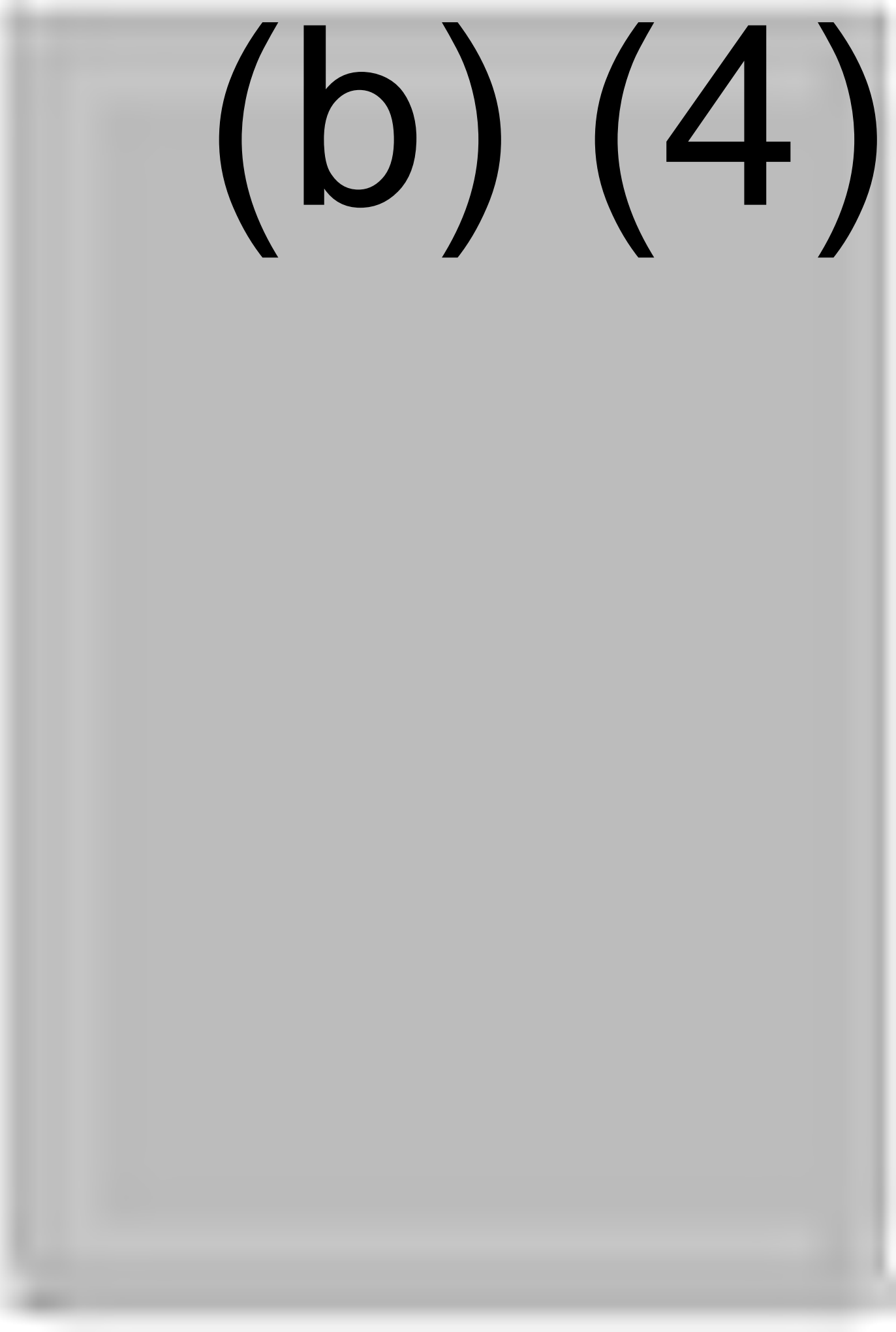
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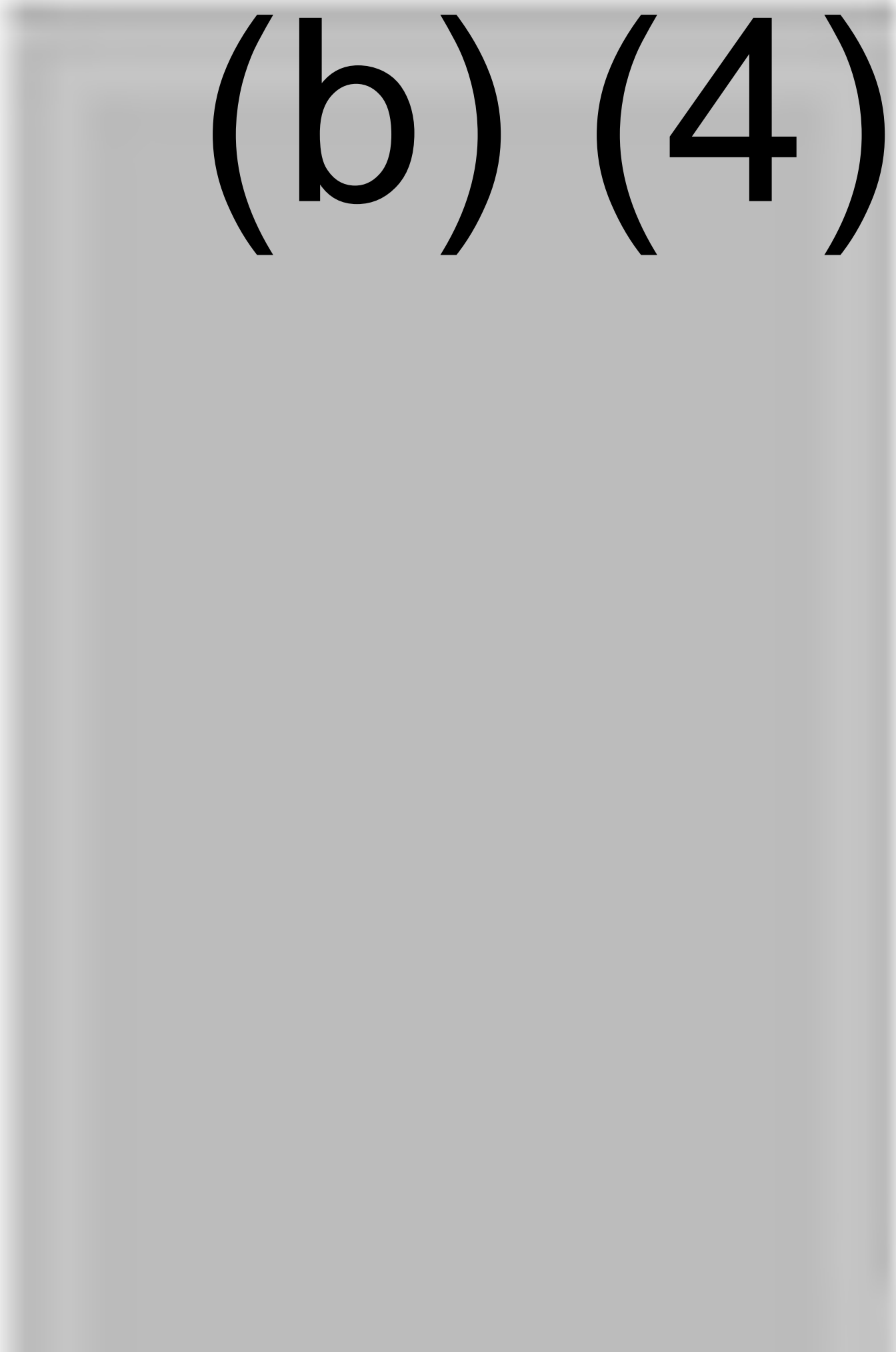
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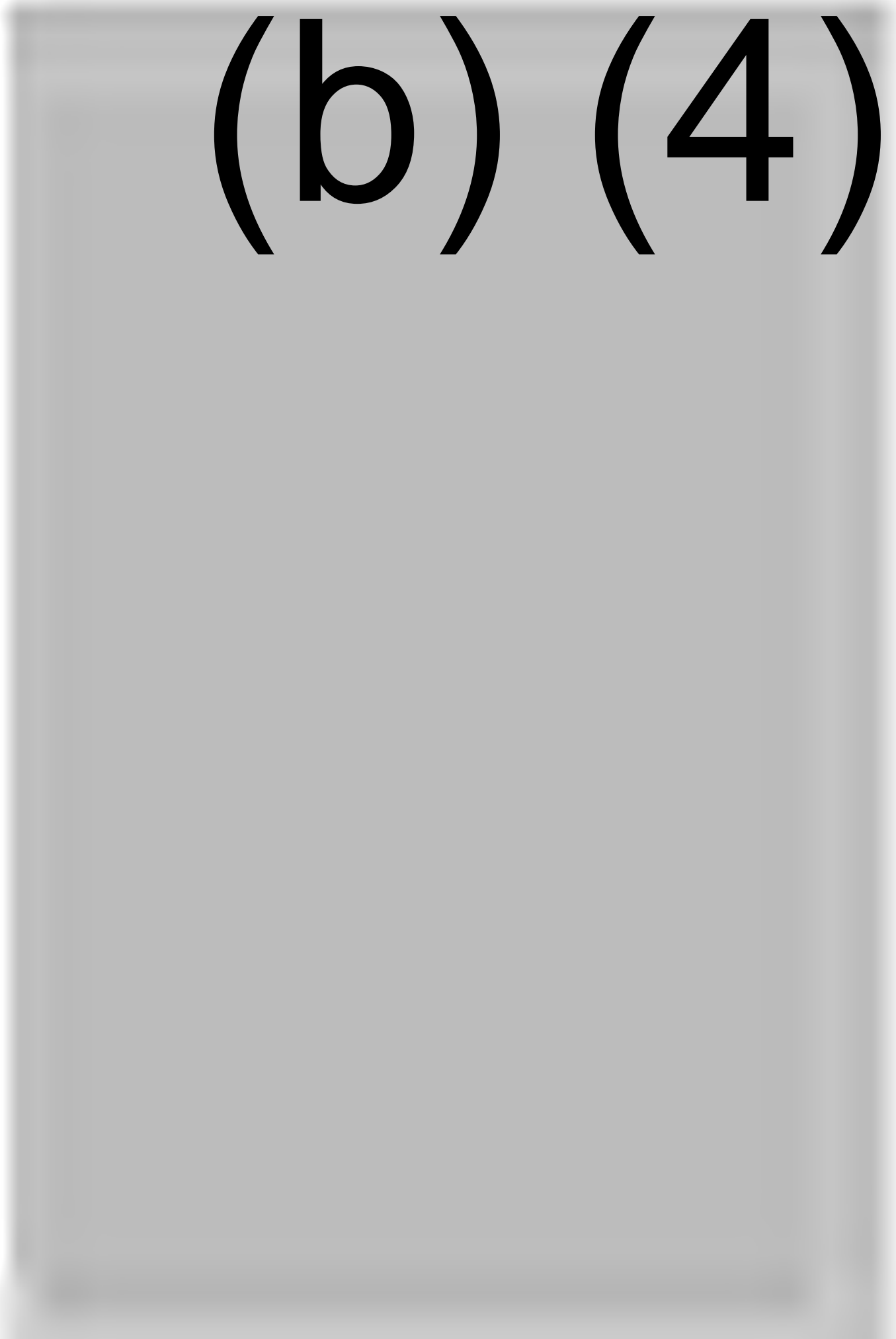
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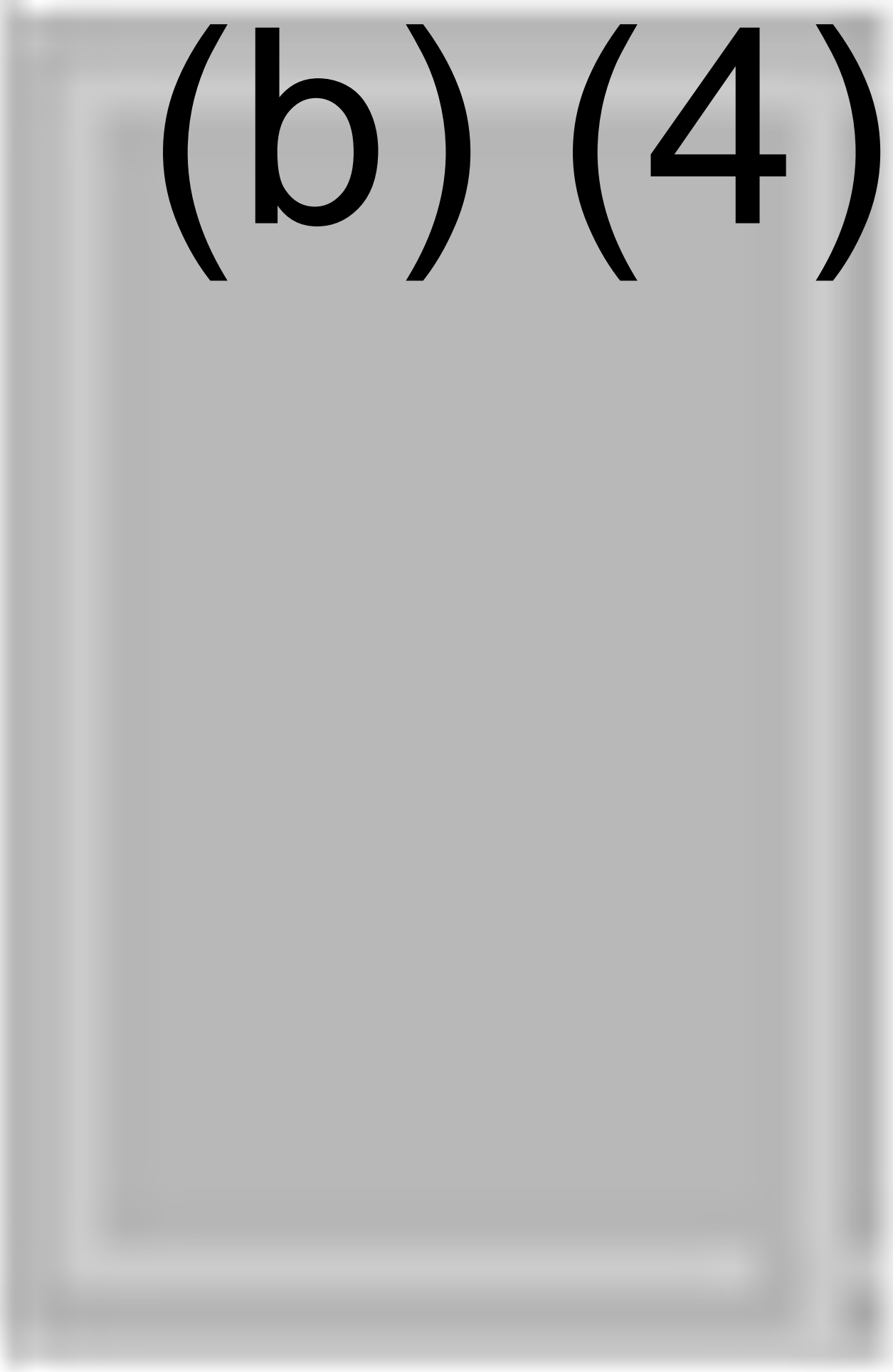
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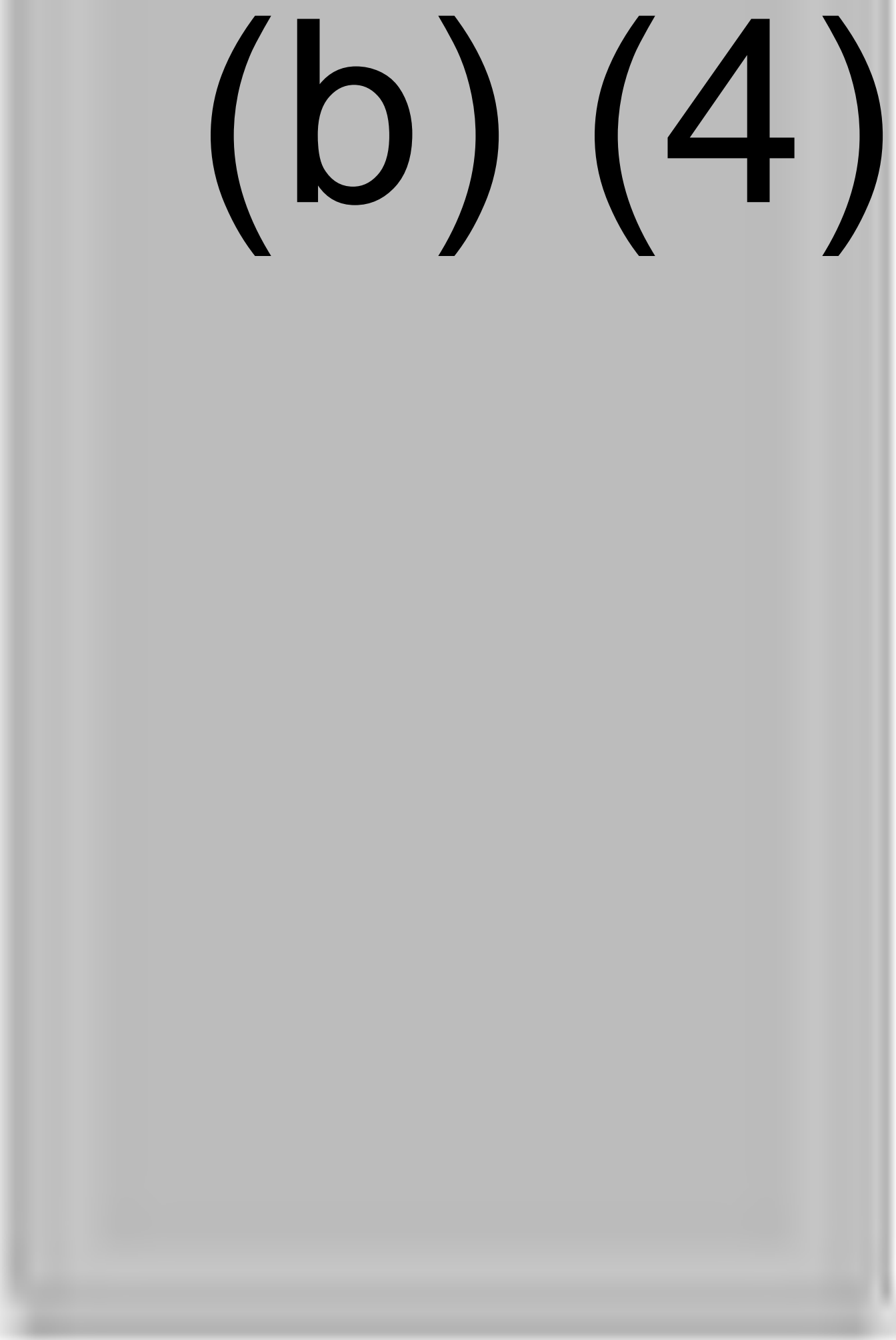
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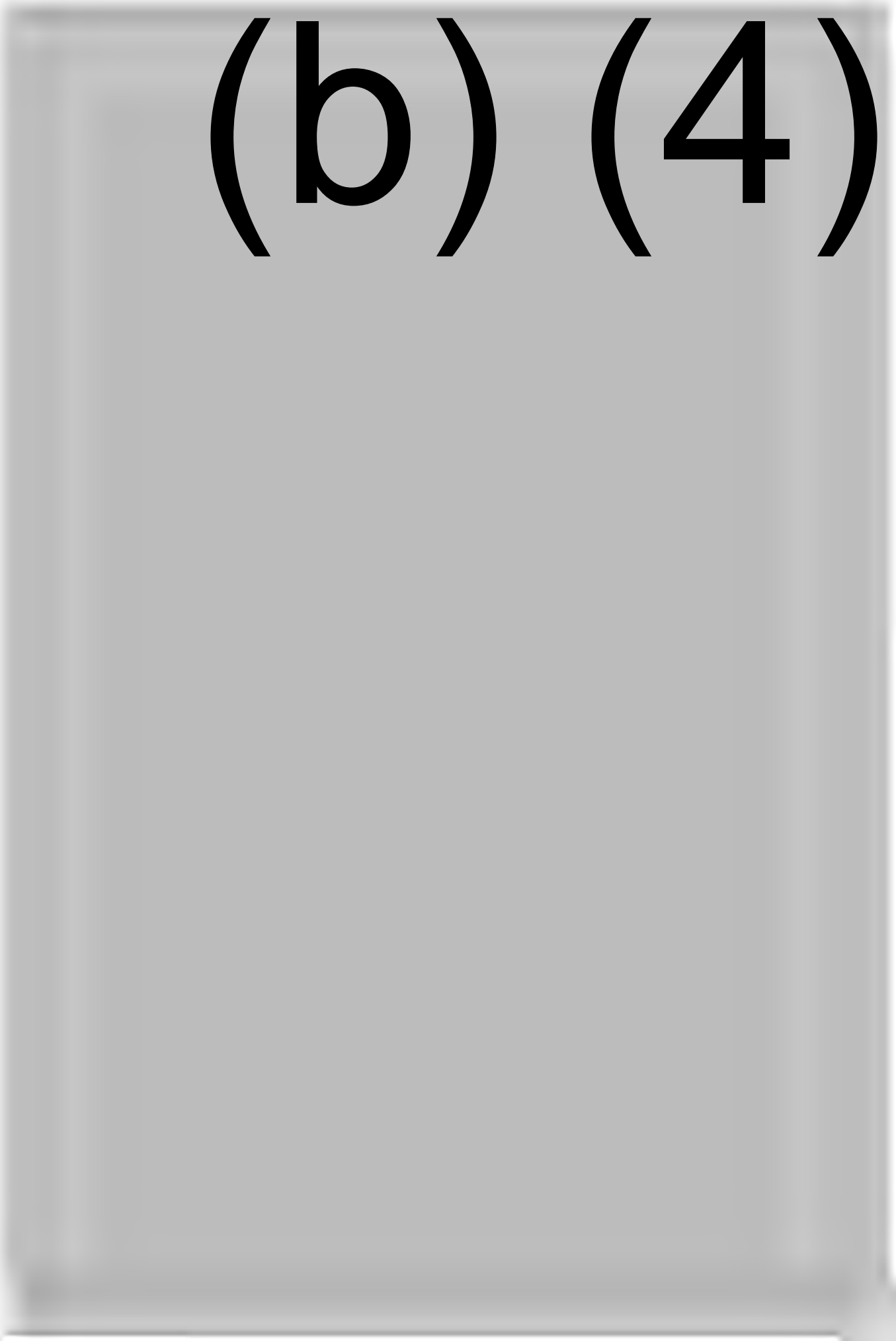
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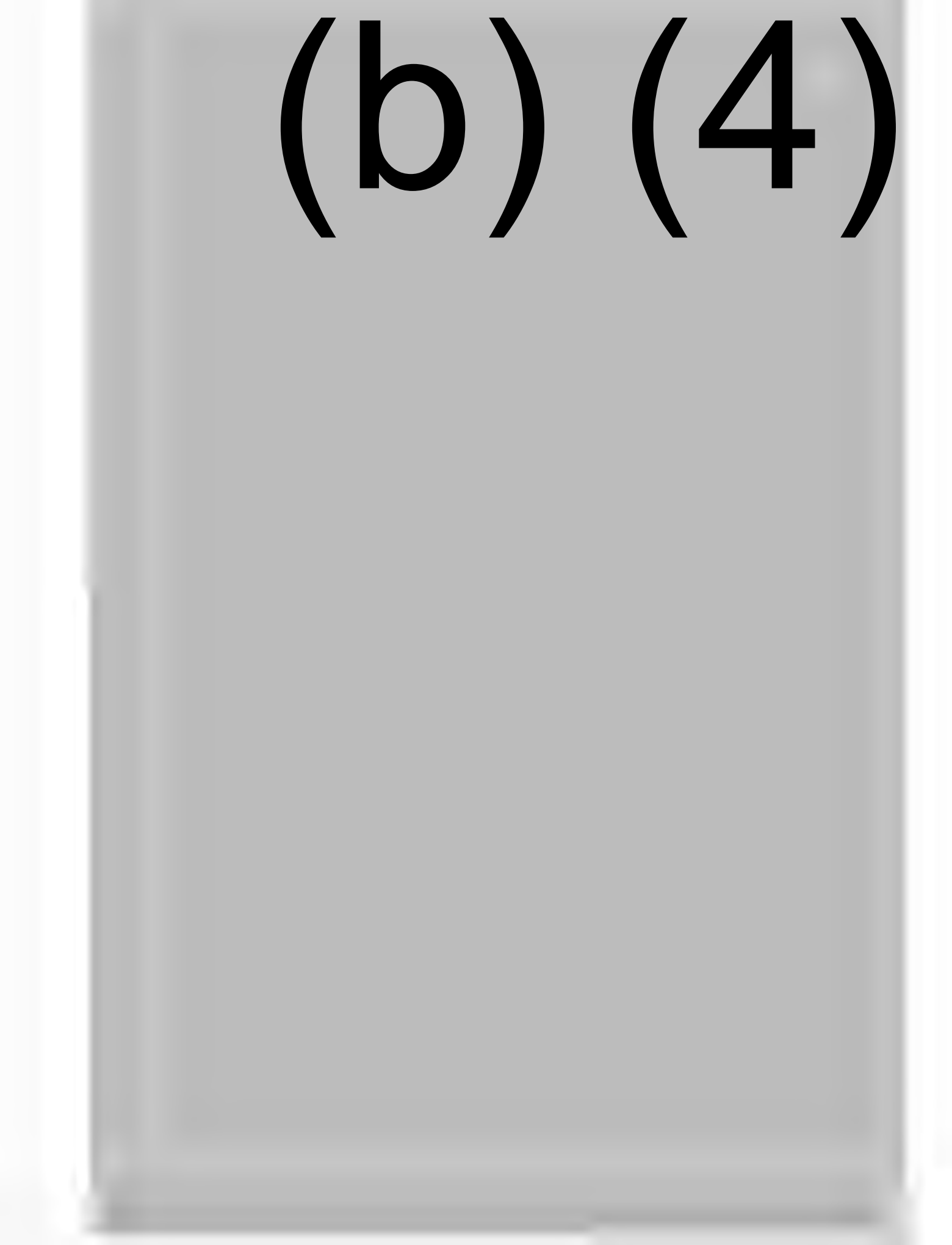
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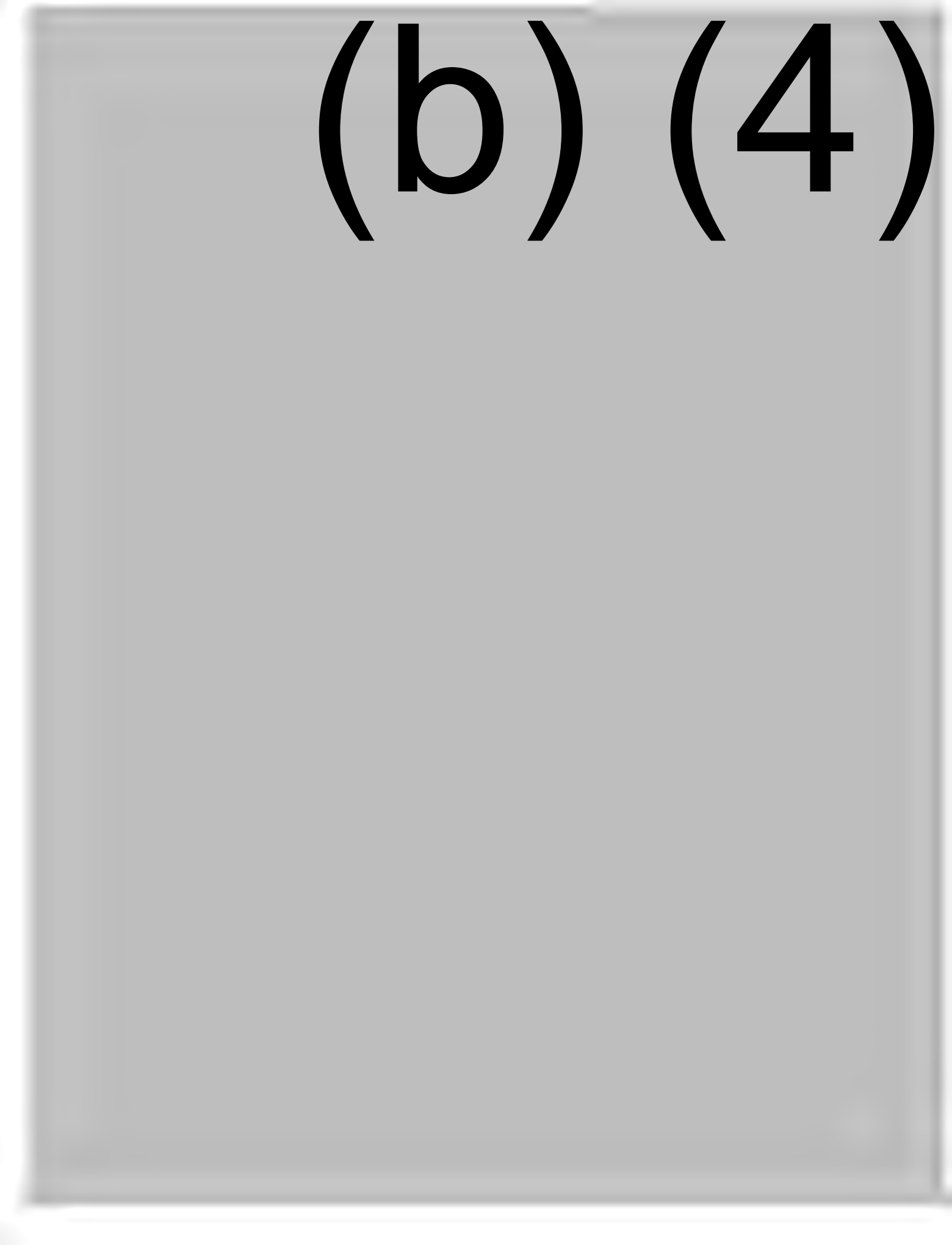
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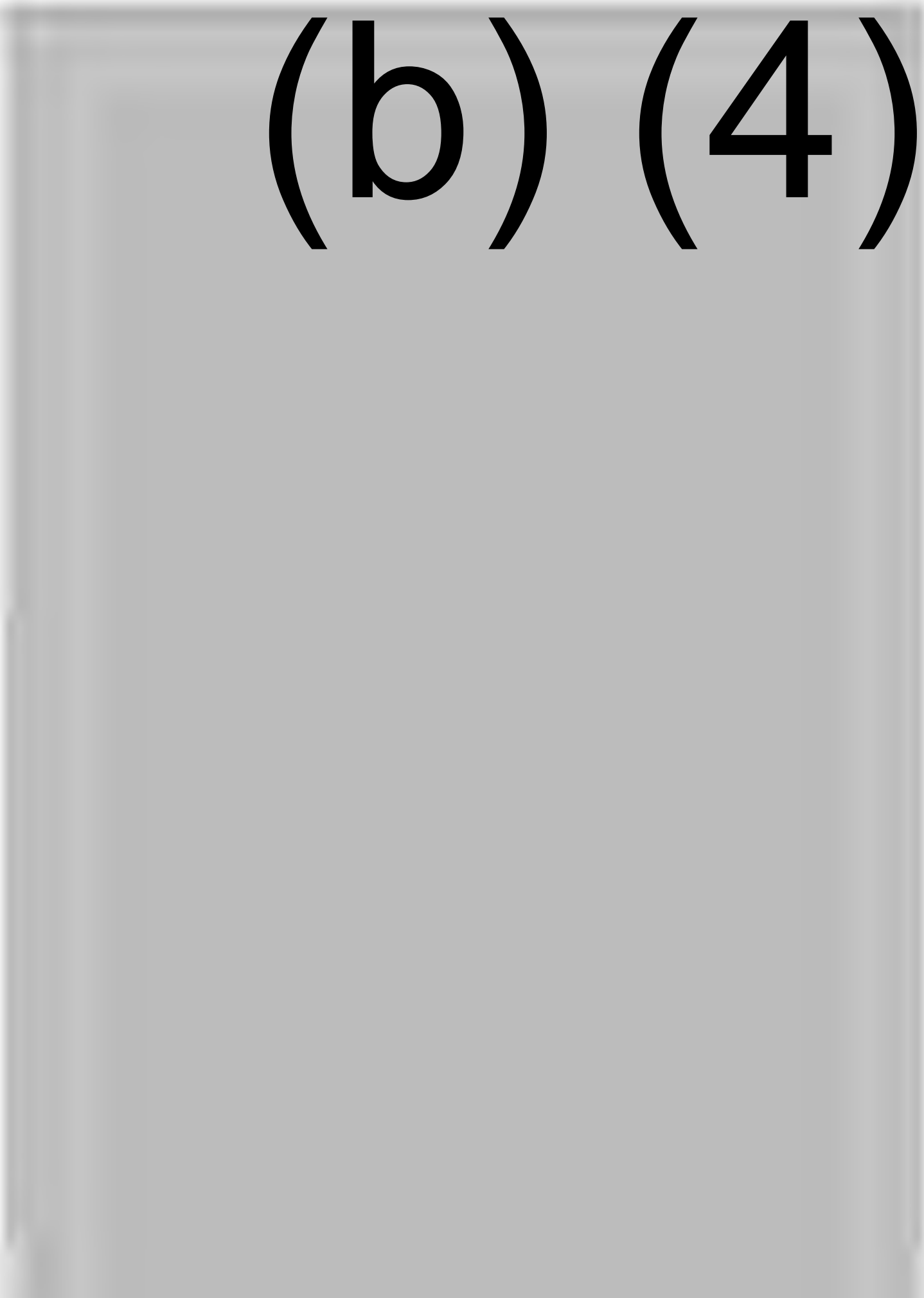
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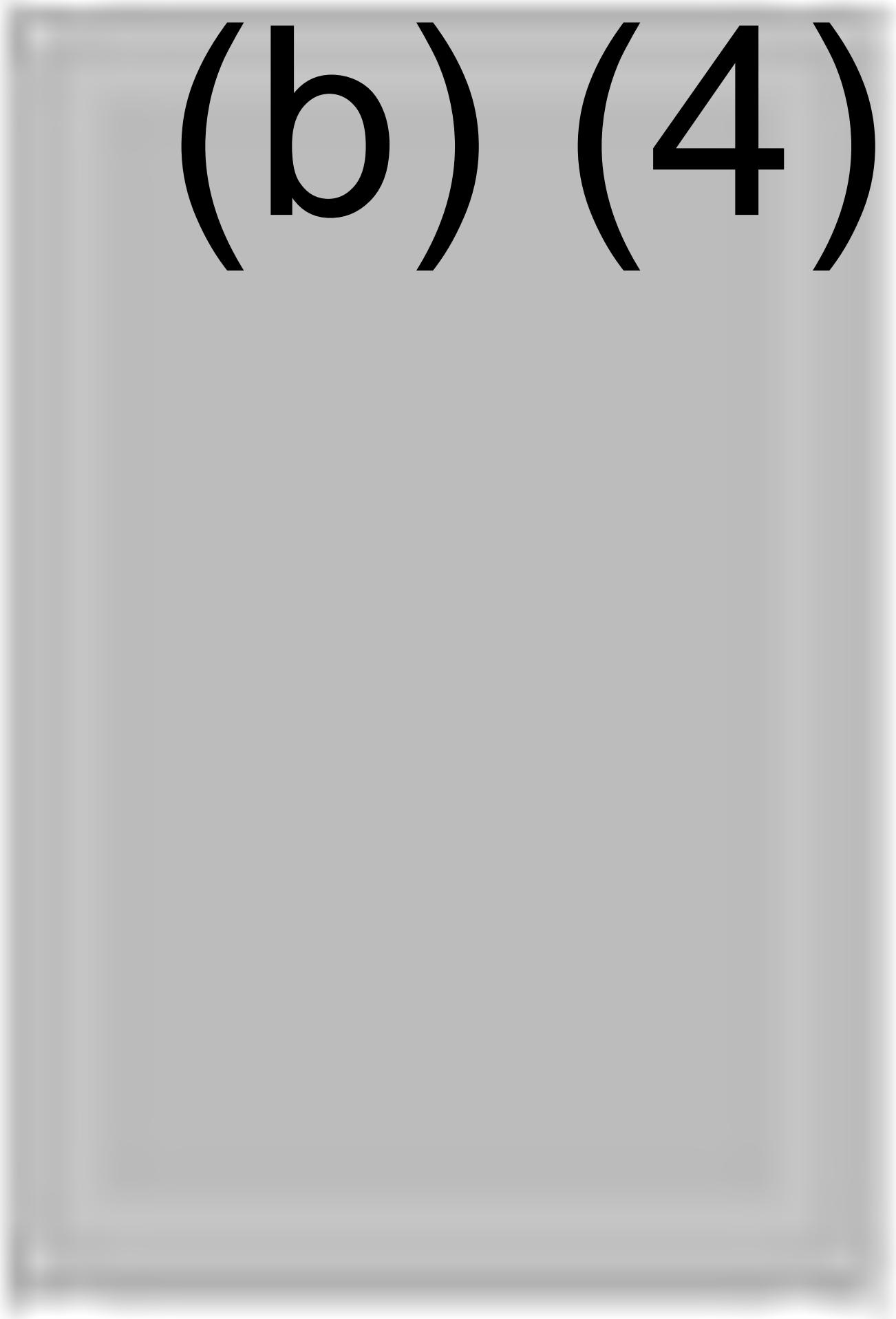
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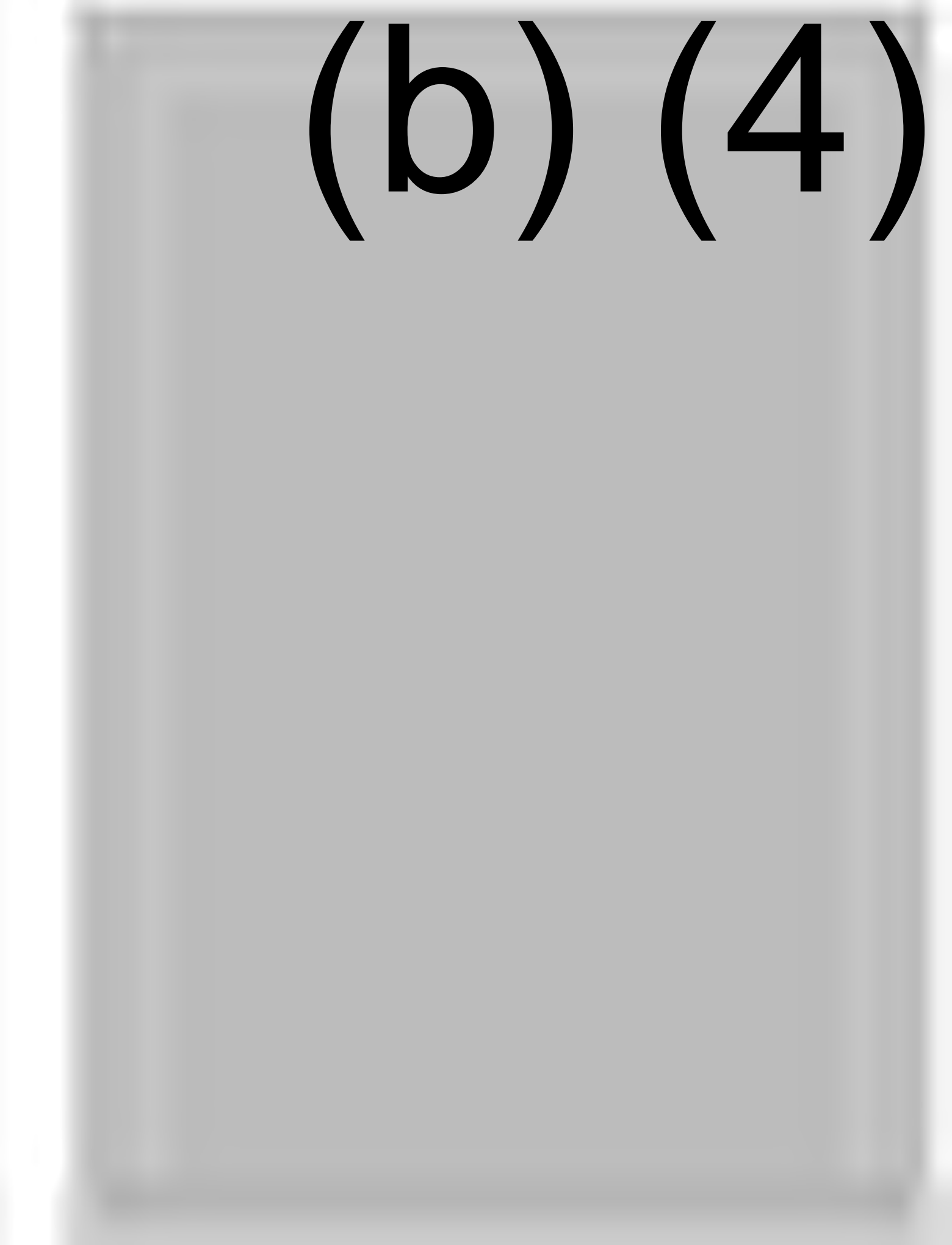
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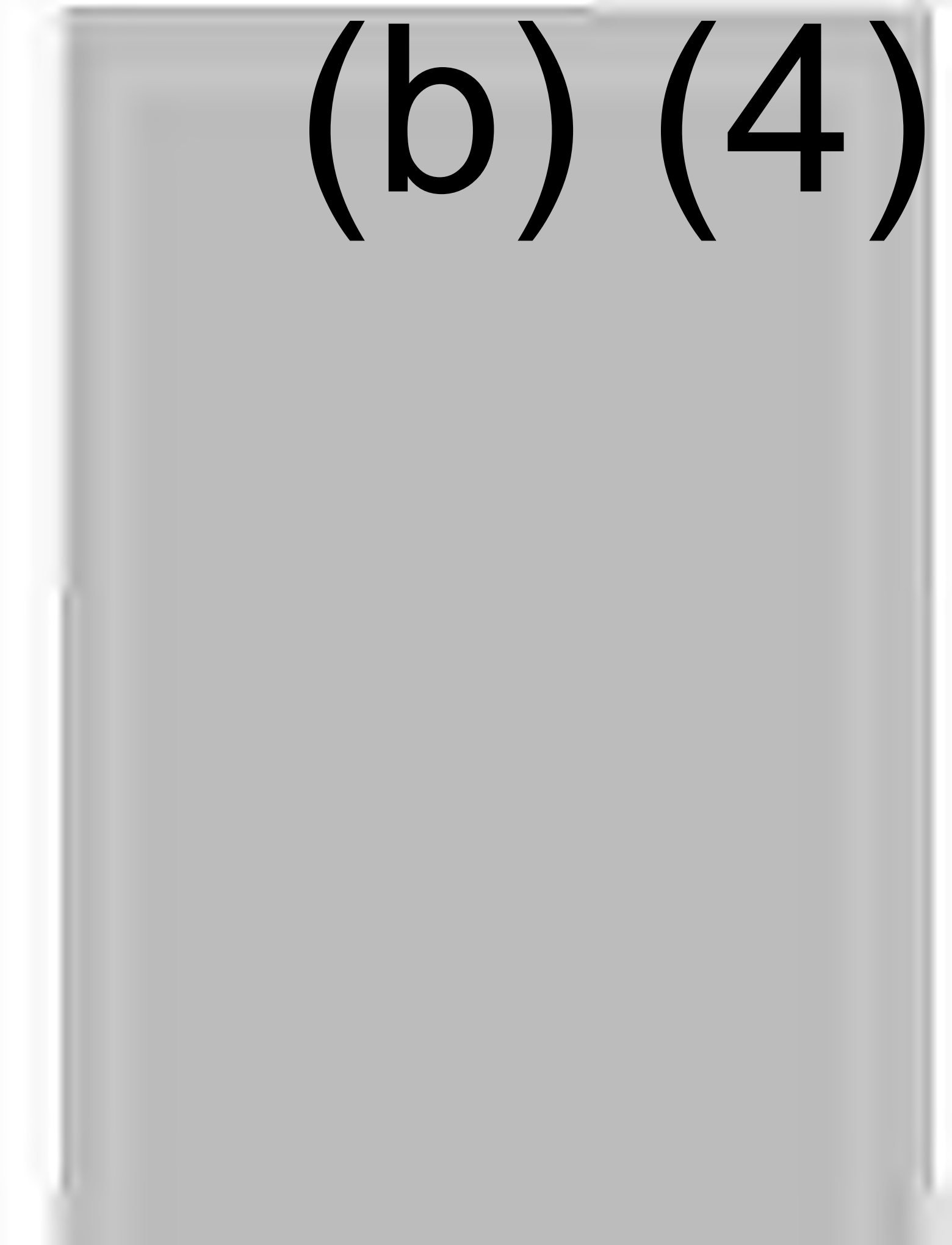
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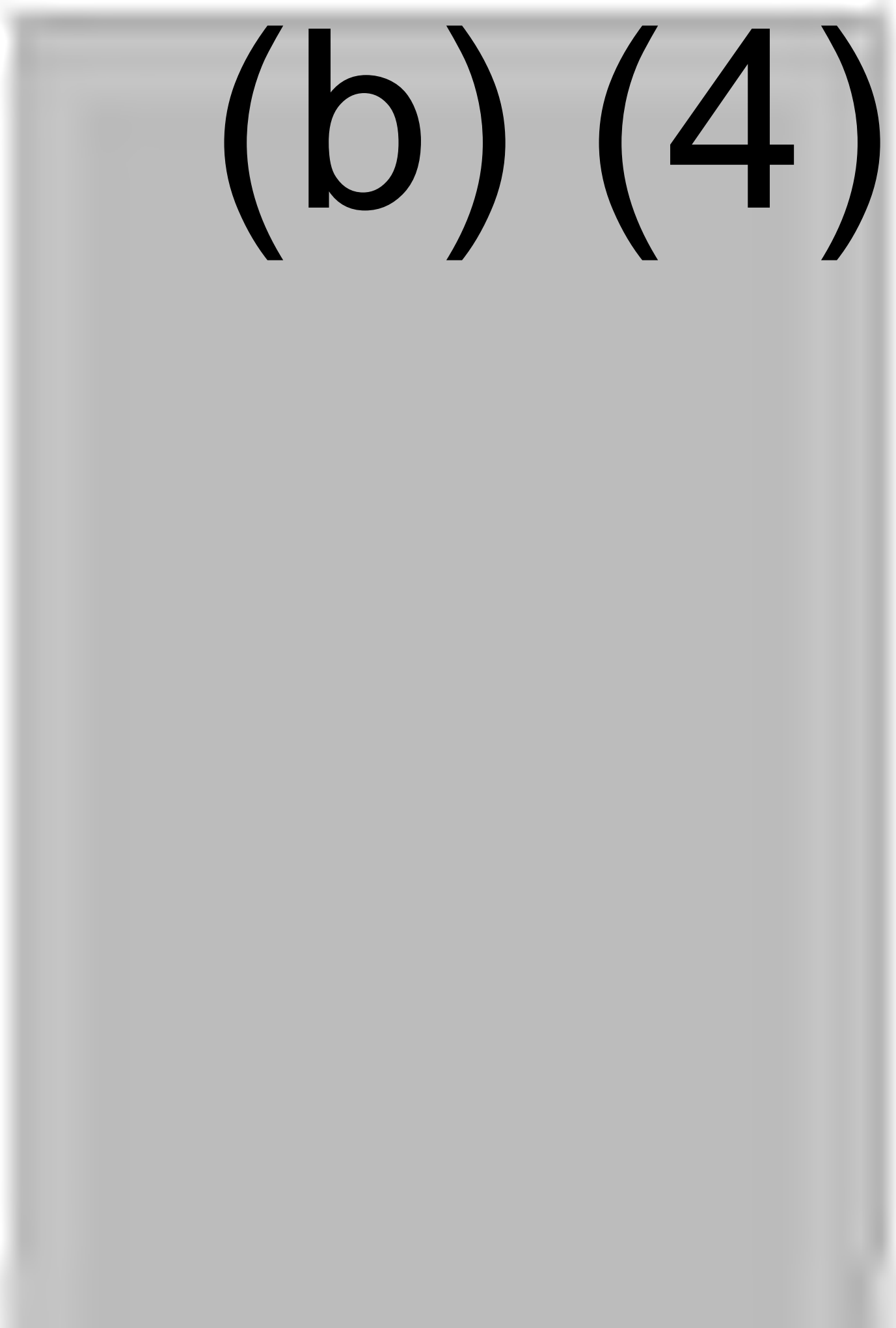
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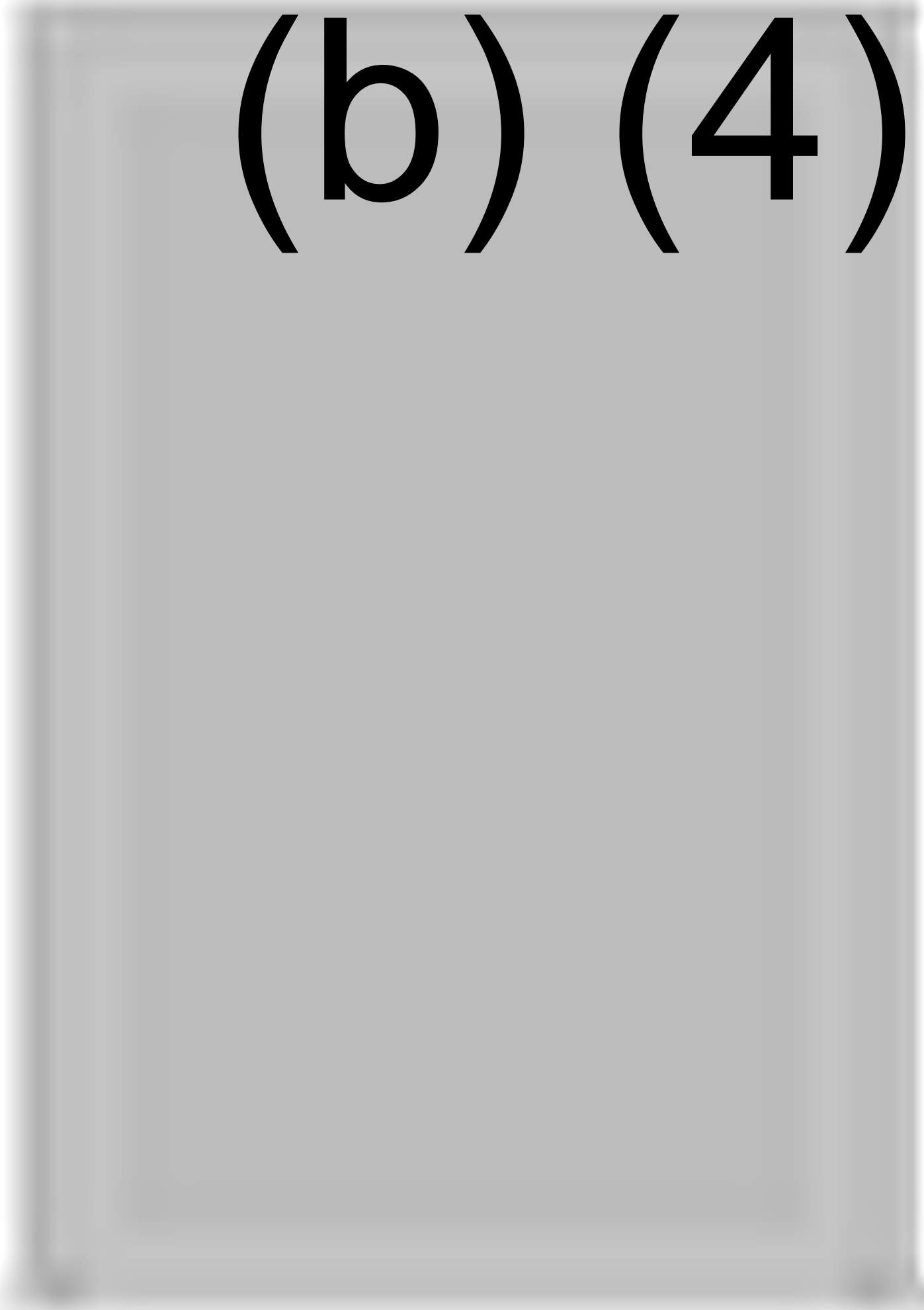
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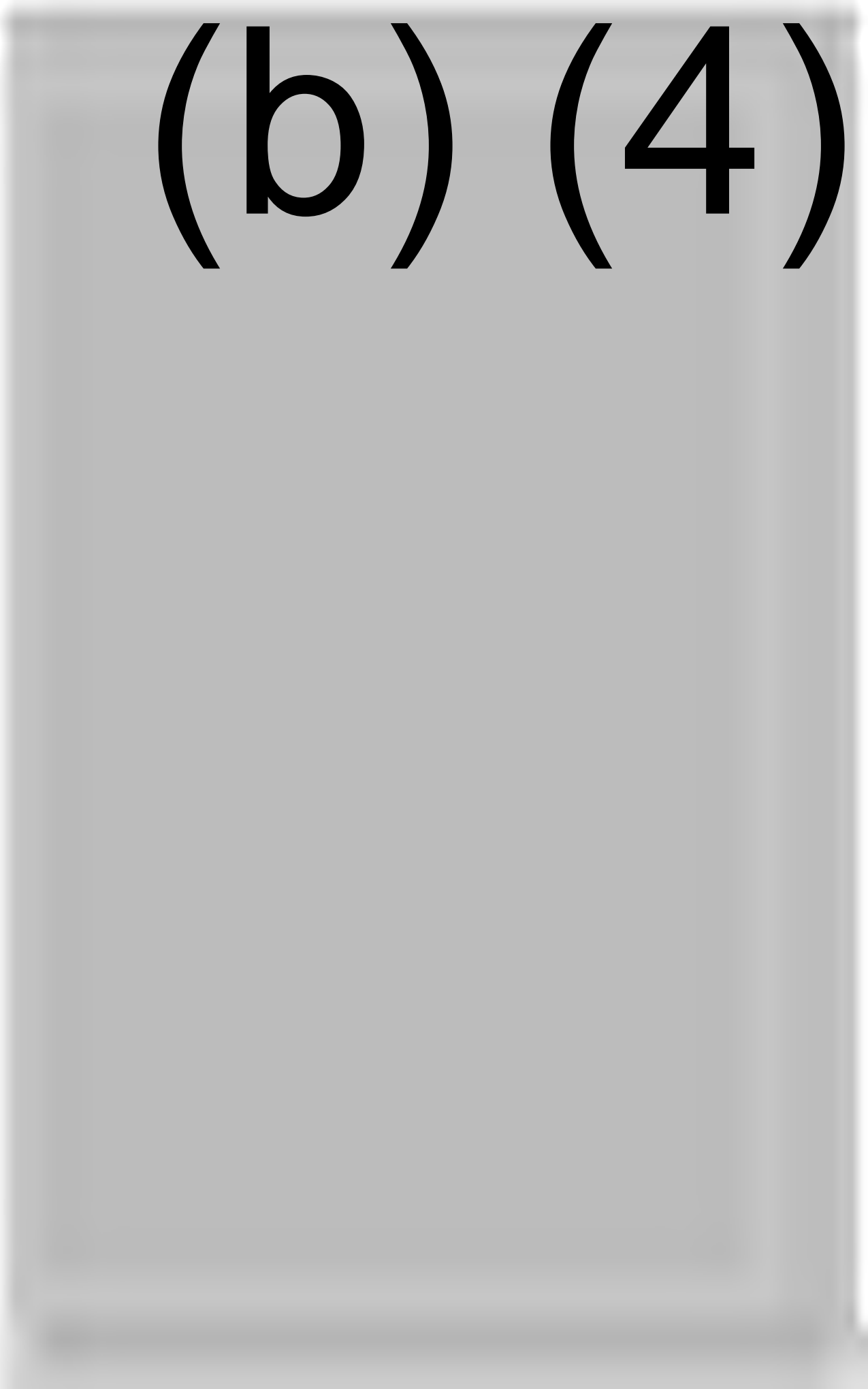
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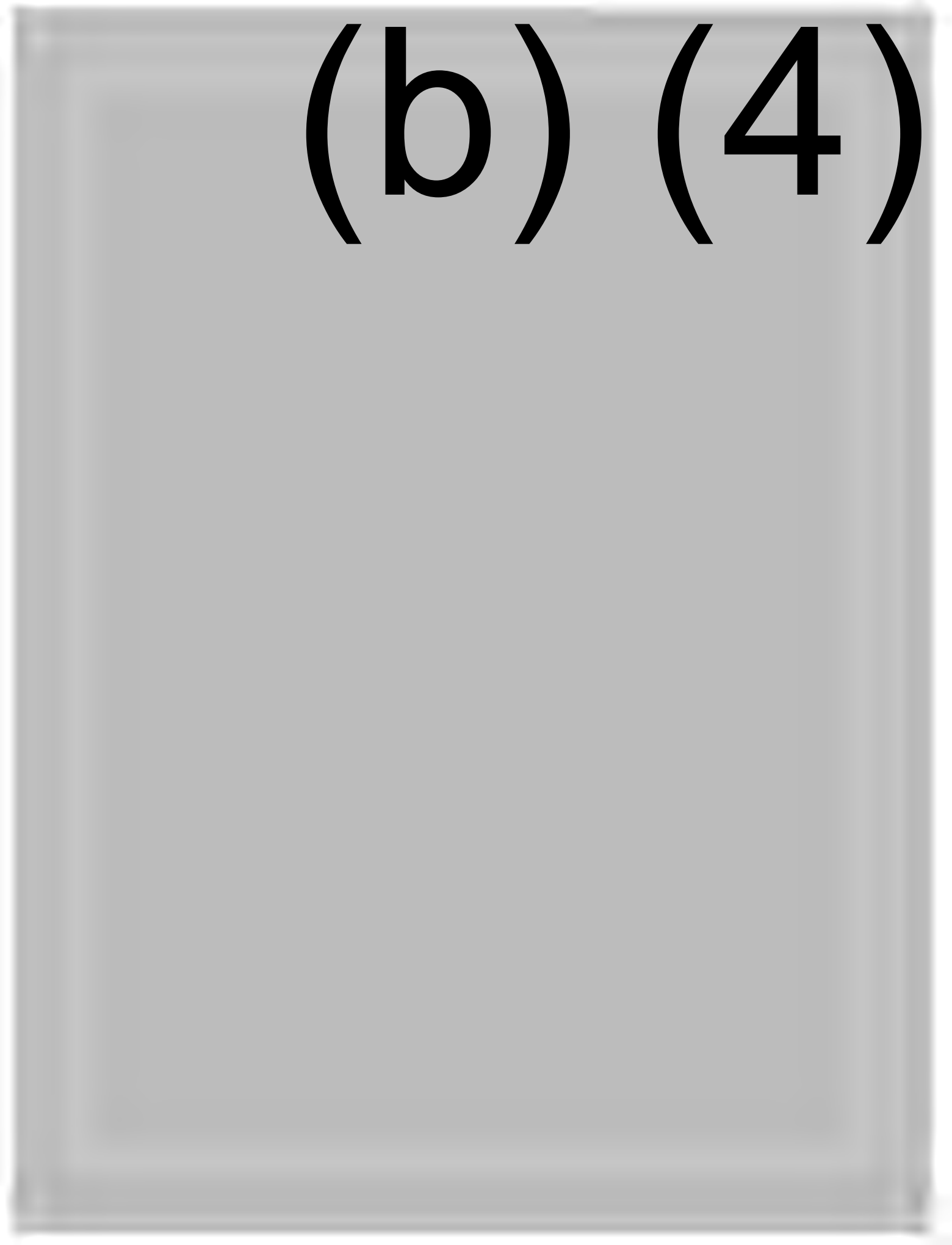
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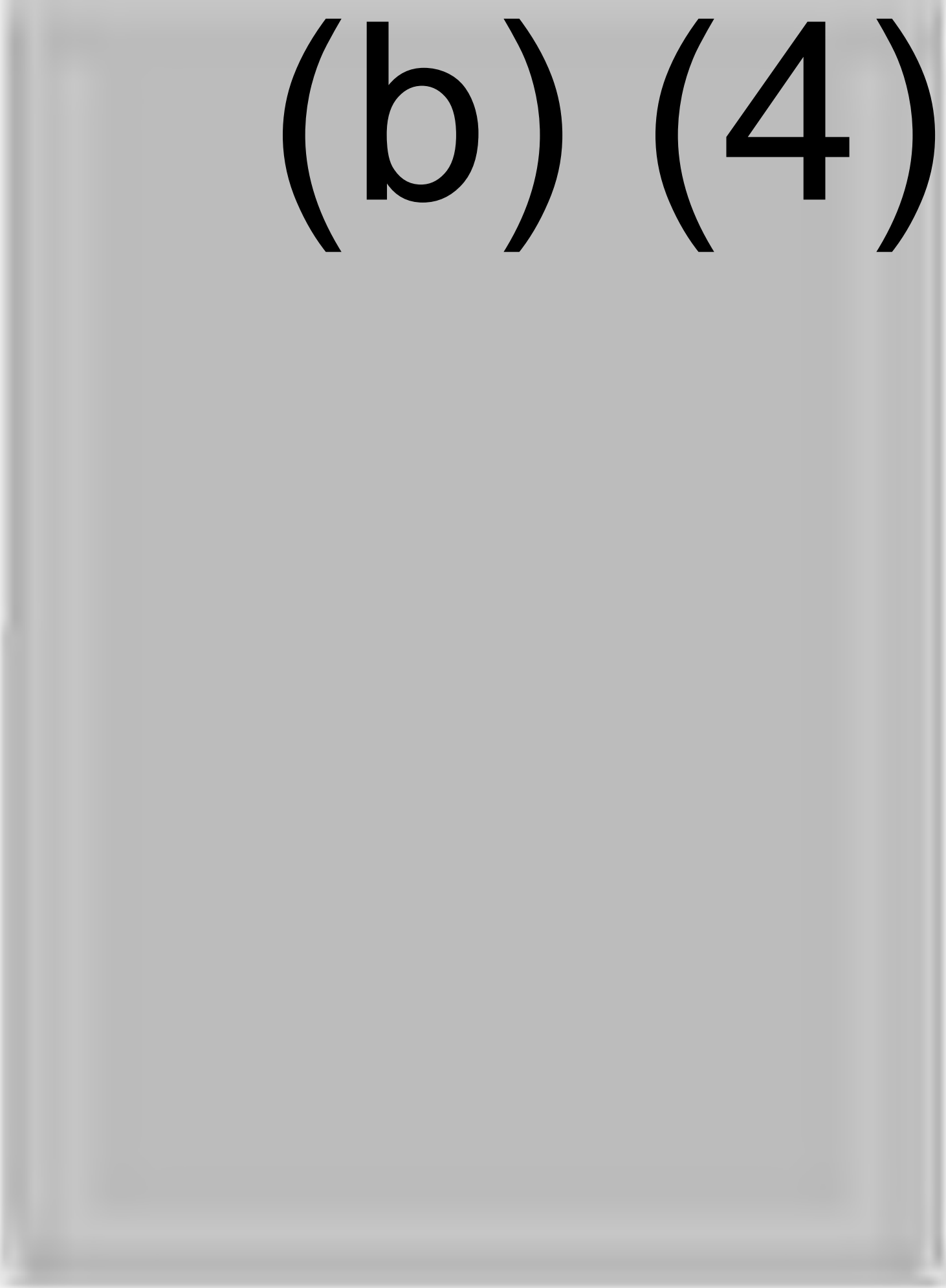
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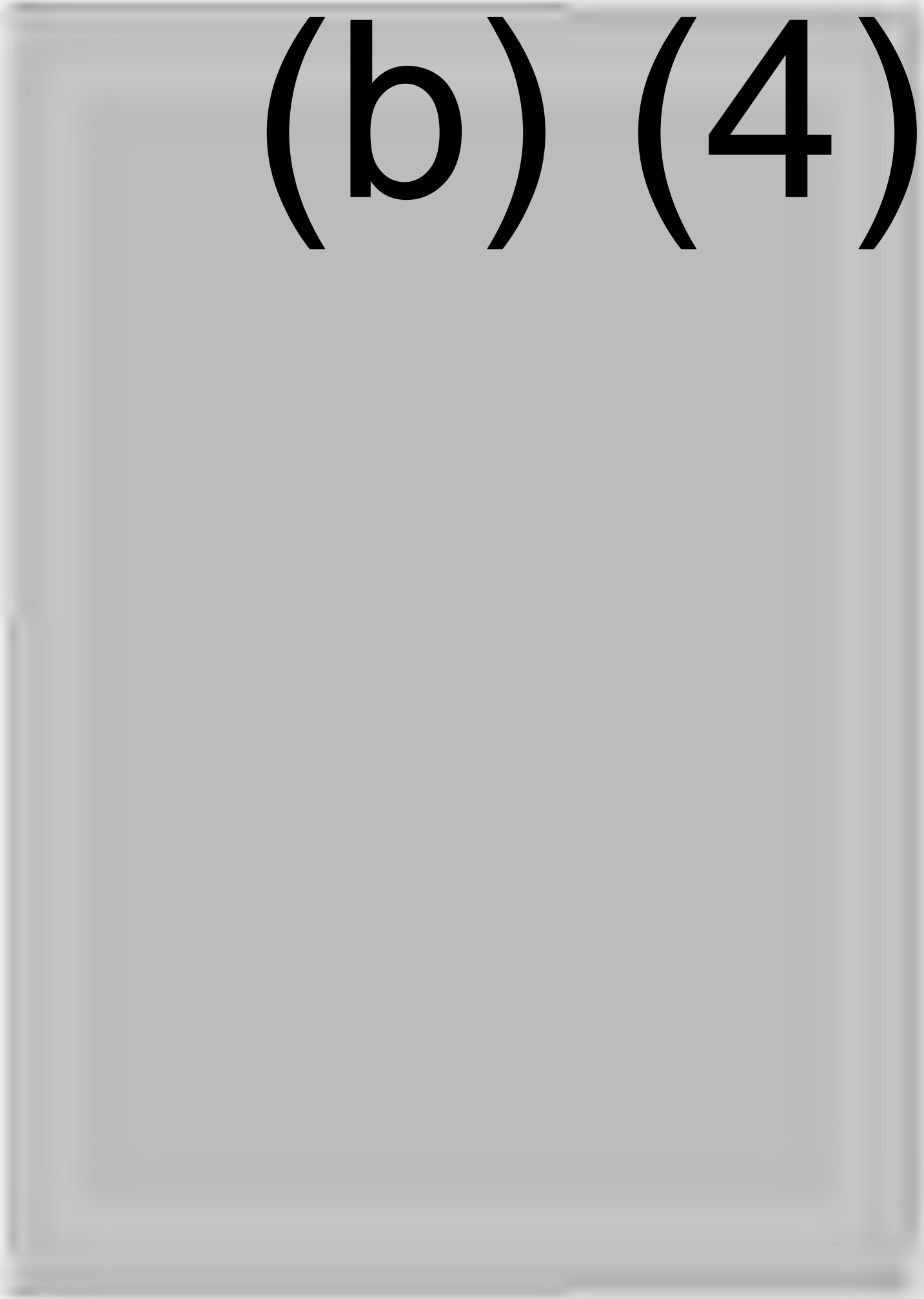
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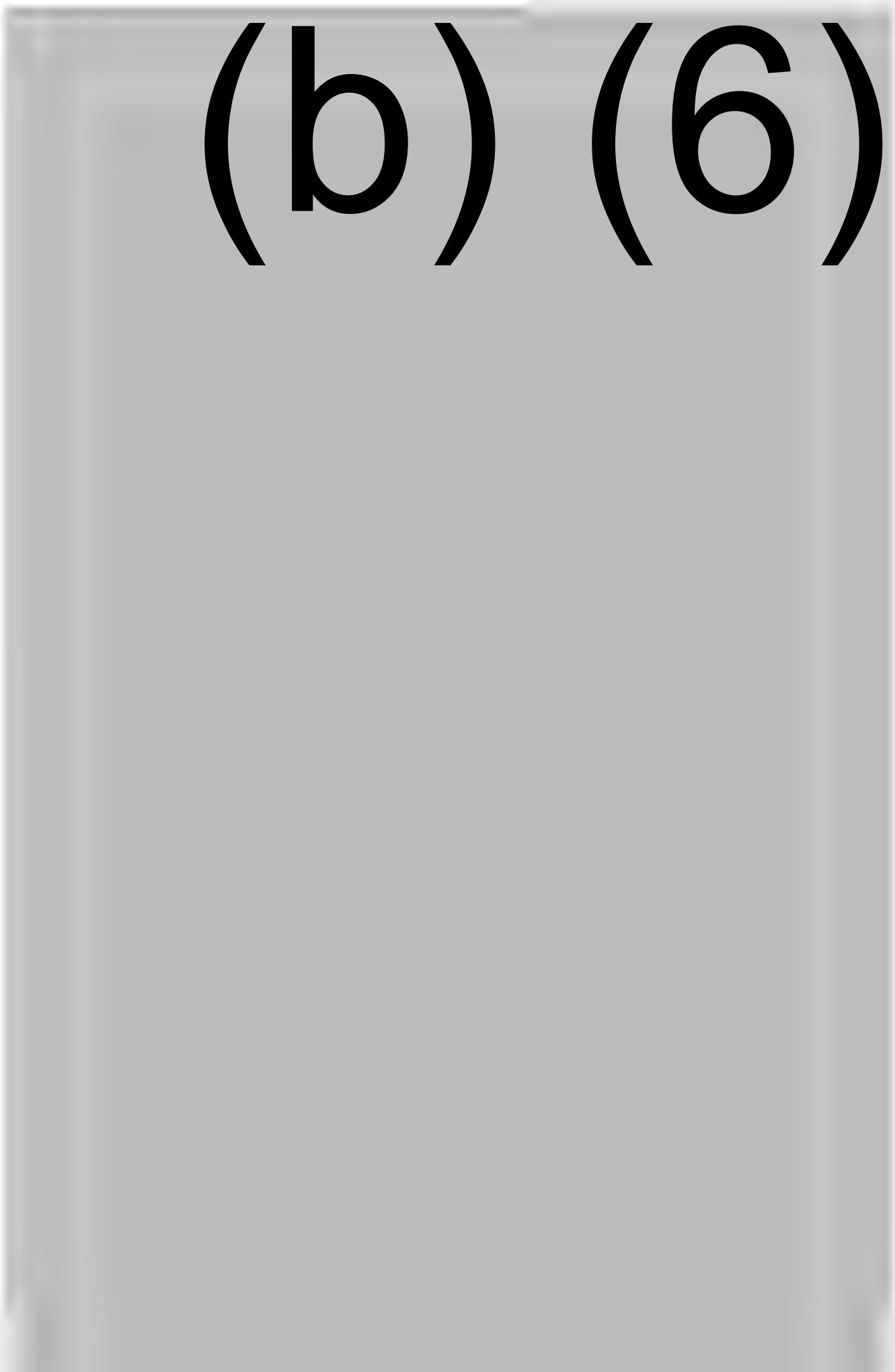
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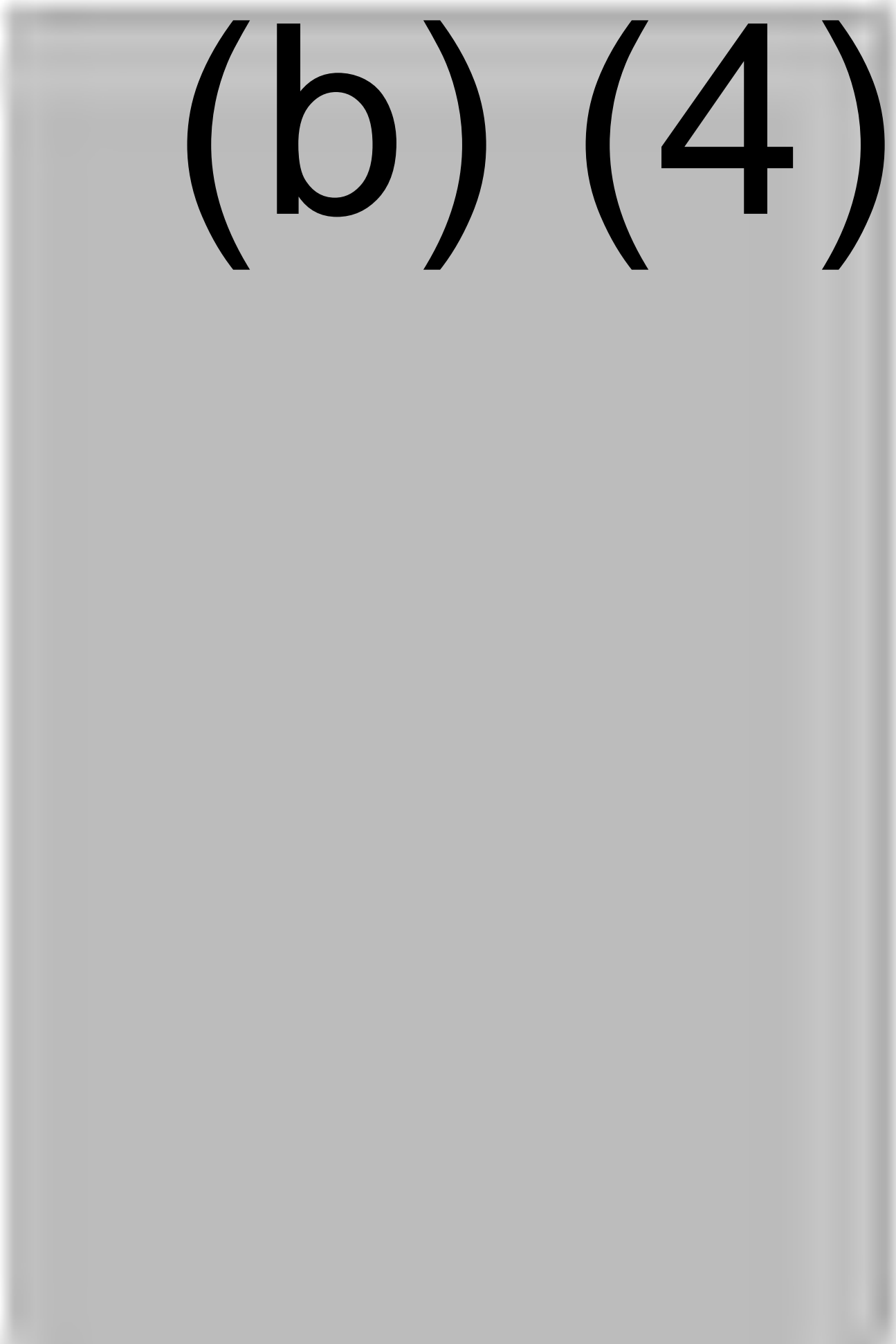
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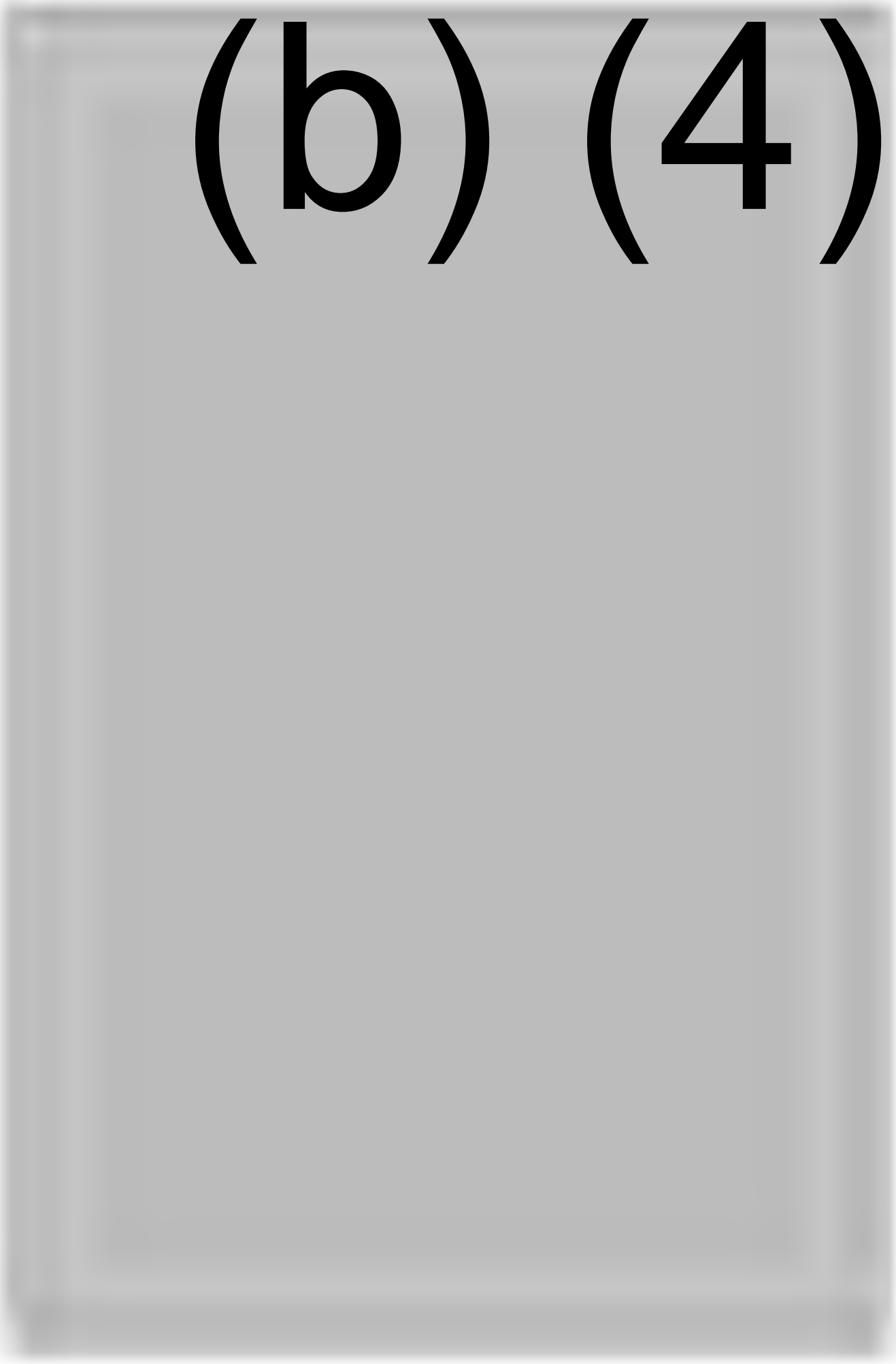
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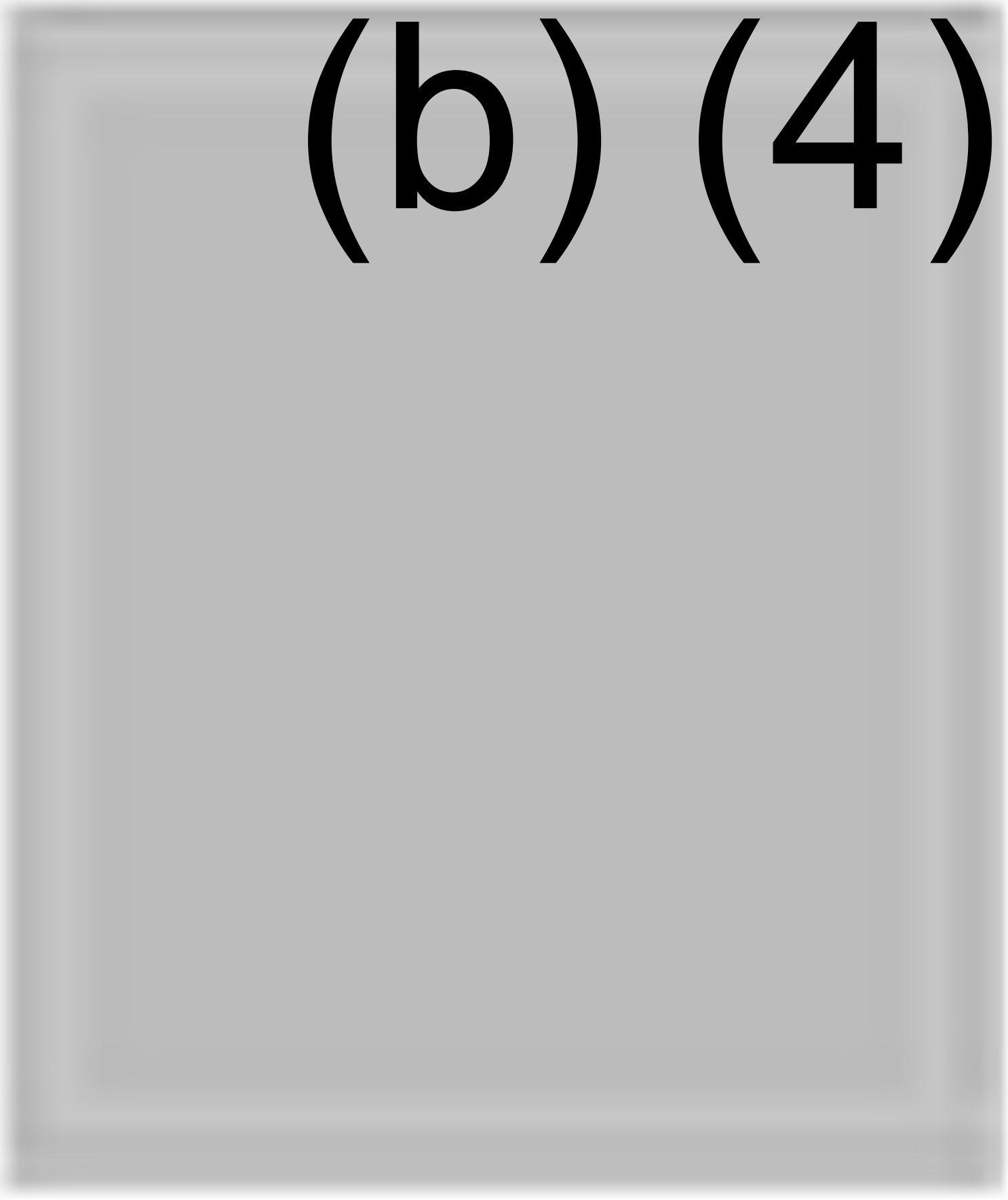
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BAM: Aerobic Plate Count

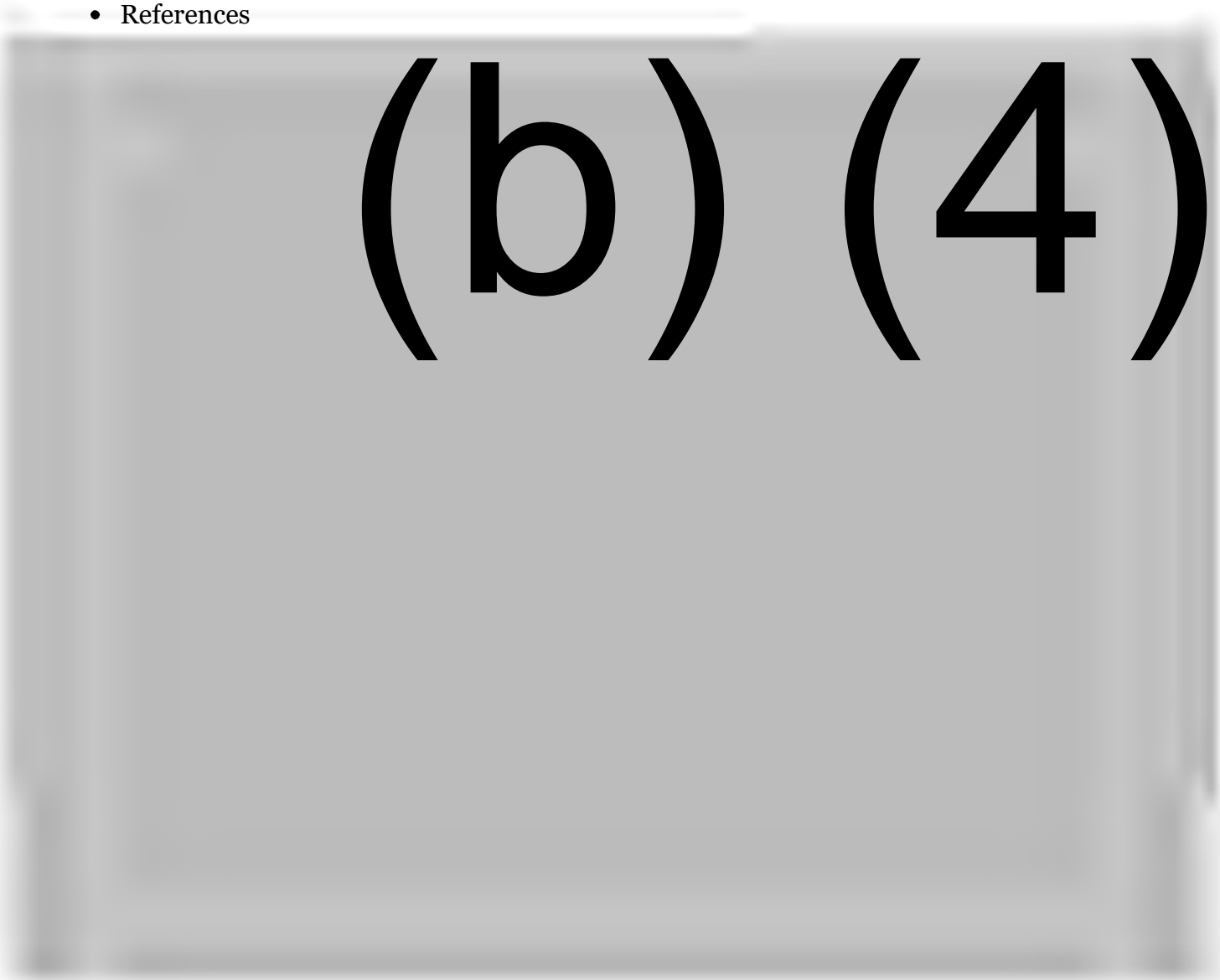
January 2001

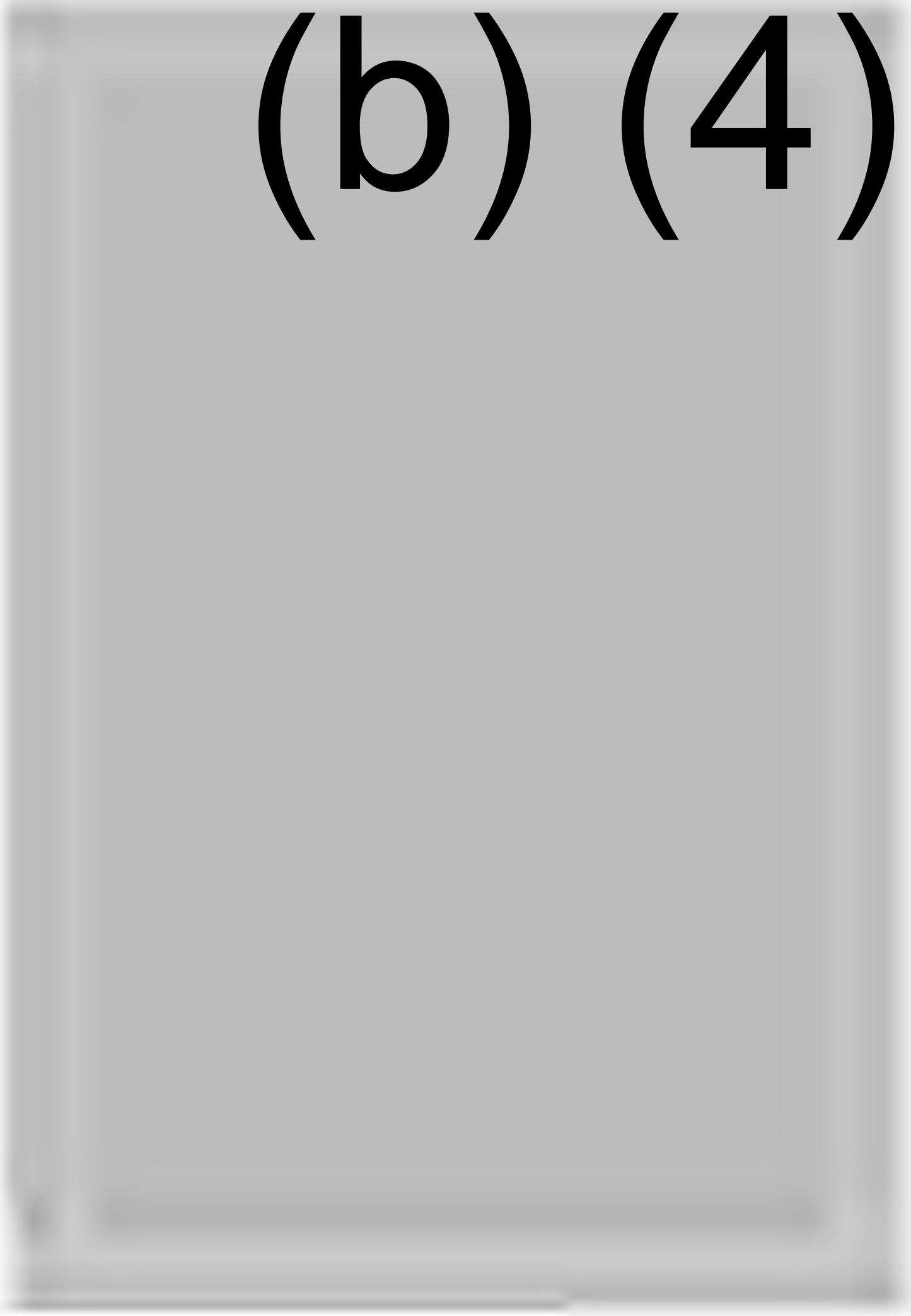
Bacteriological Analytical Manual
Chapter 3
Aerobic Plate Count

Authors: Larry Maturin (ret.) and James T. Peeler (ret)

For additional information, contact Guodong Zhang (mailto:guodong.zhang@fda.hhs.gov).

Chapter Contents

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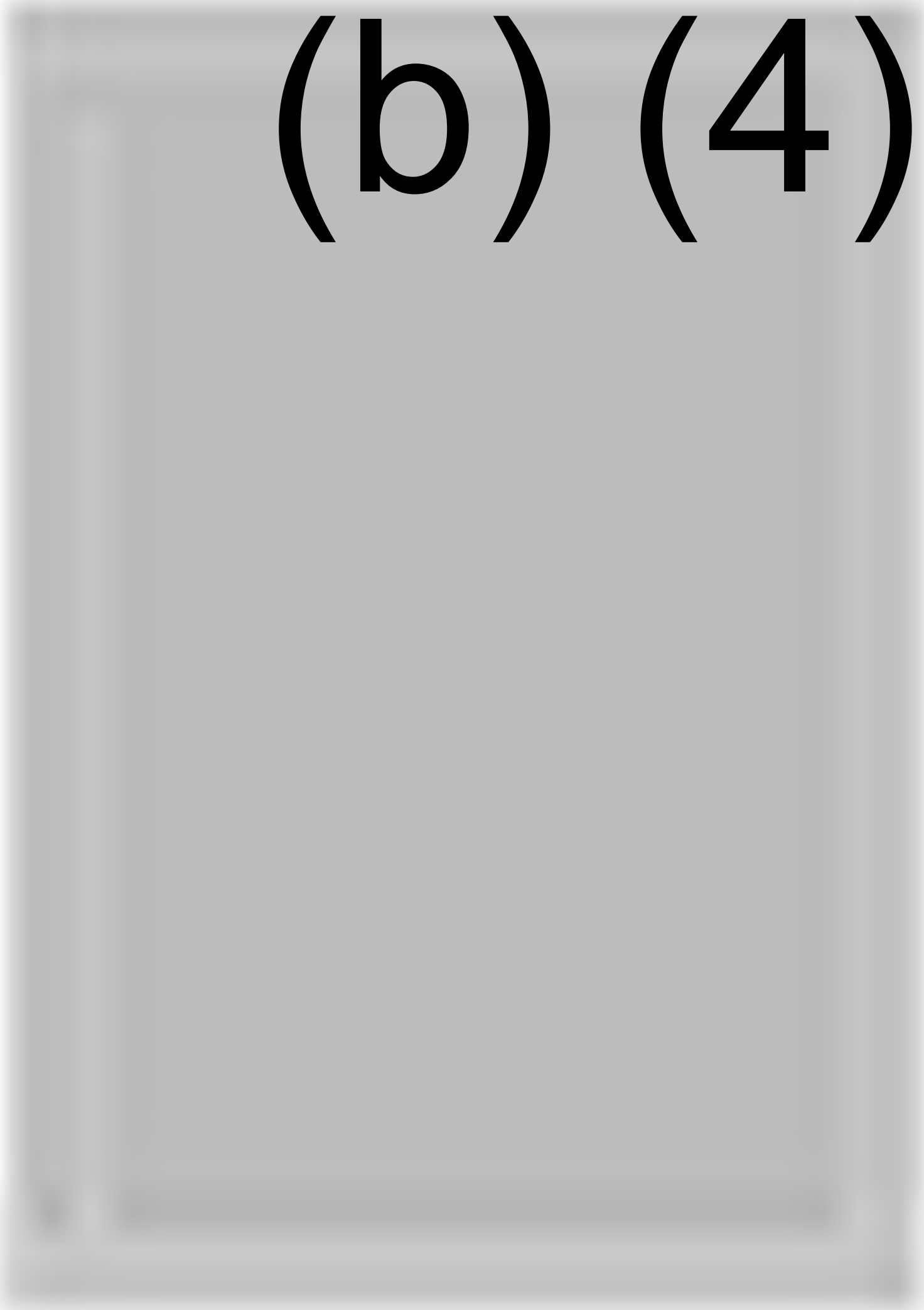
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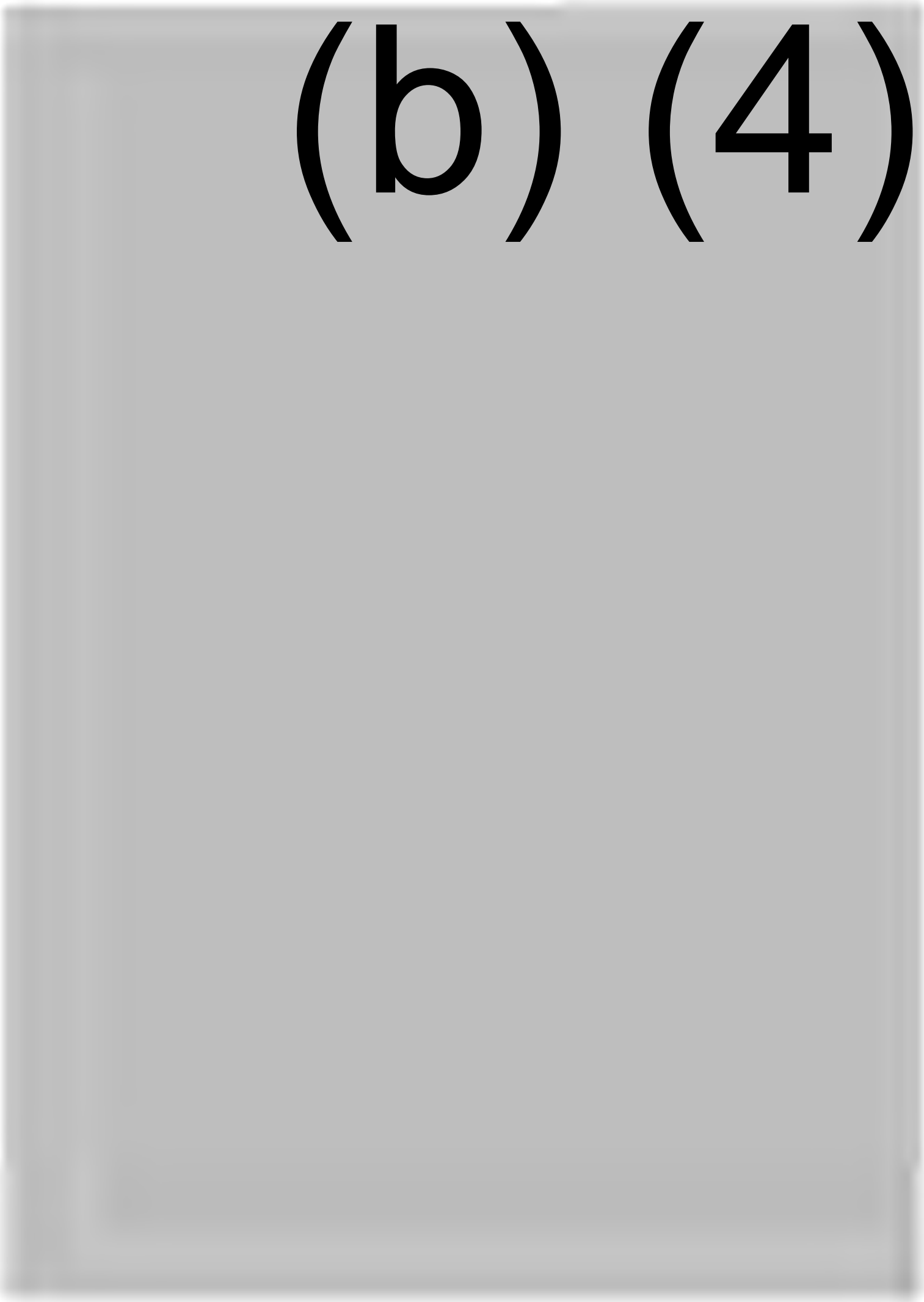
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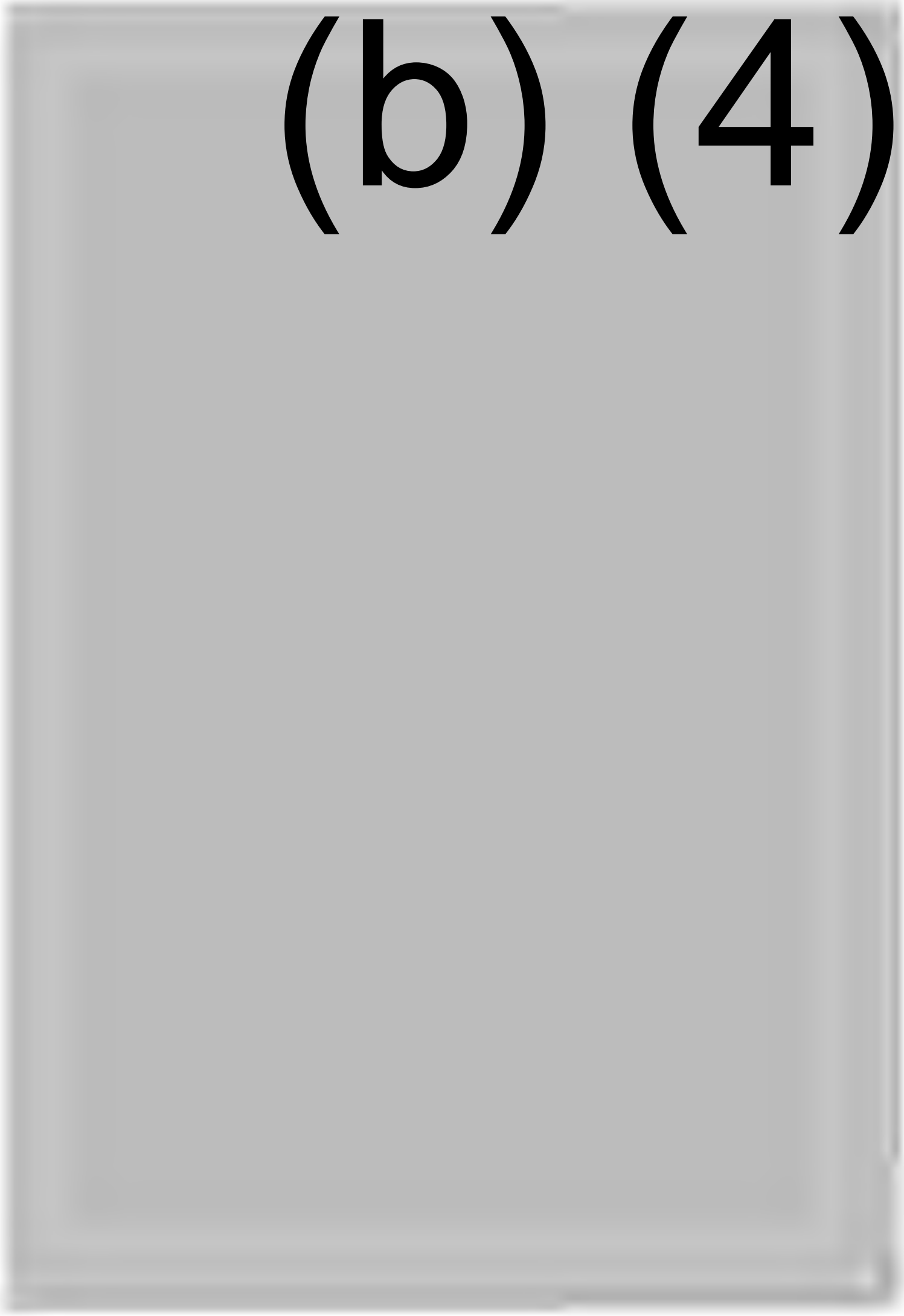
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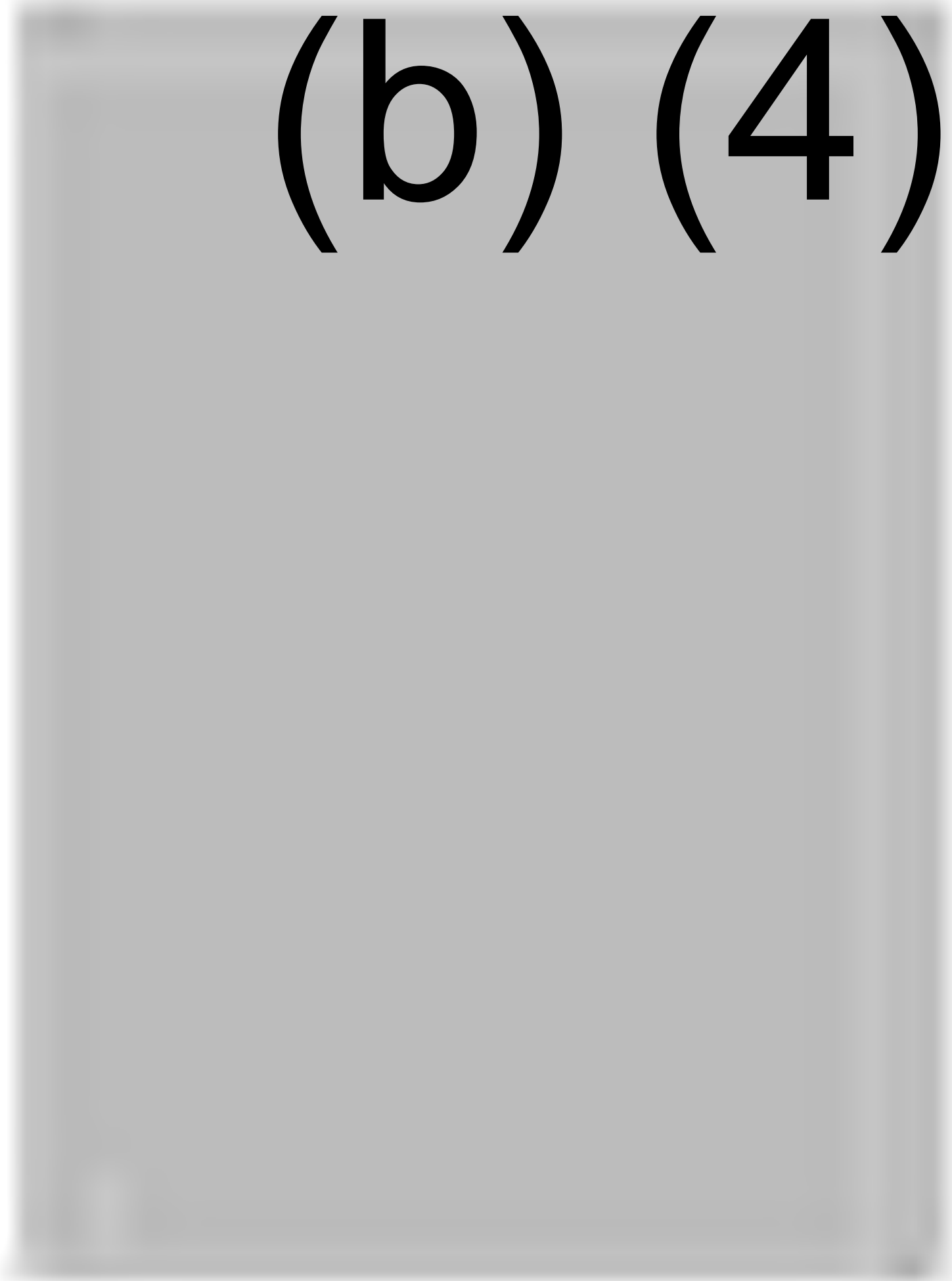
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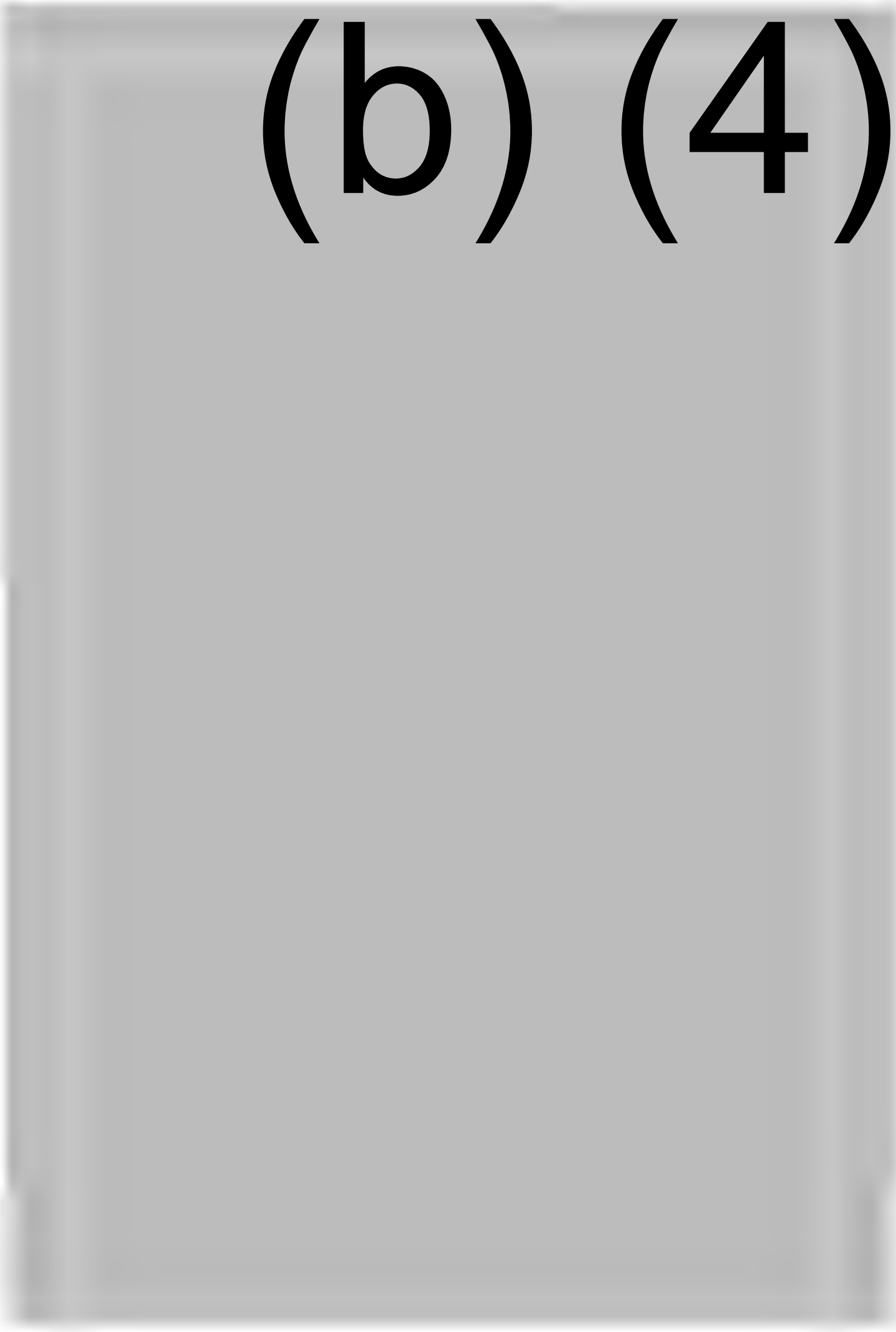
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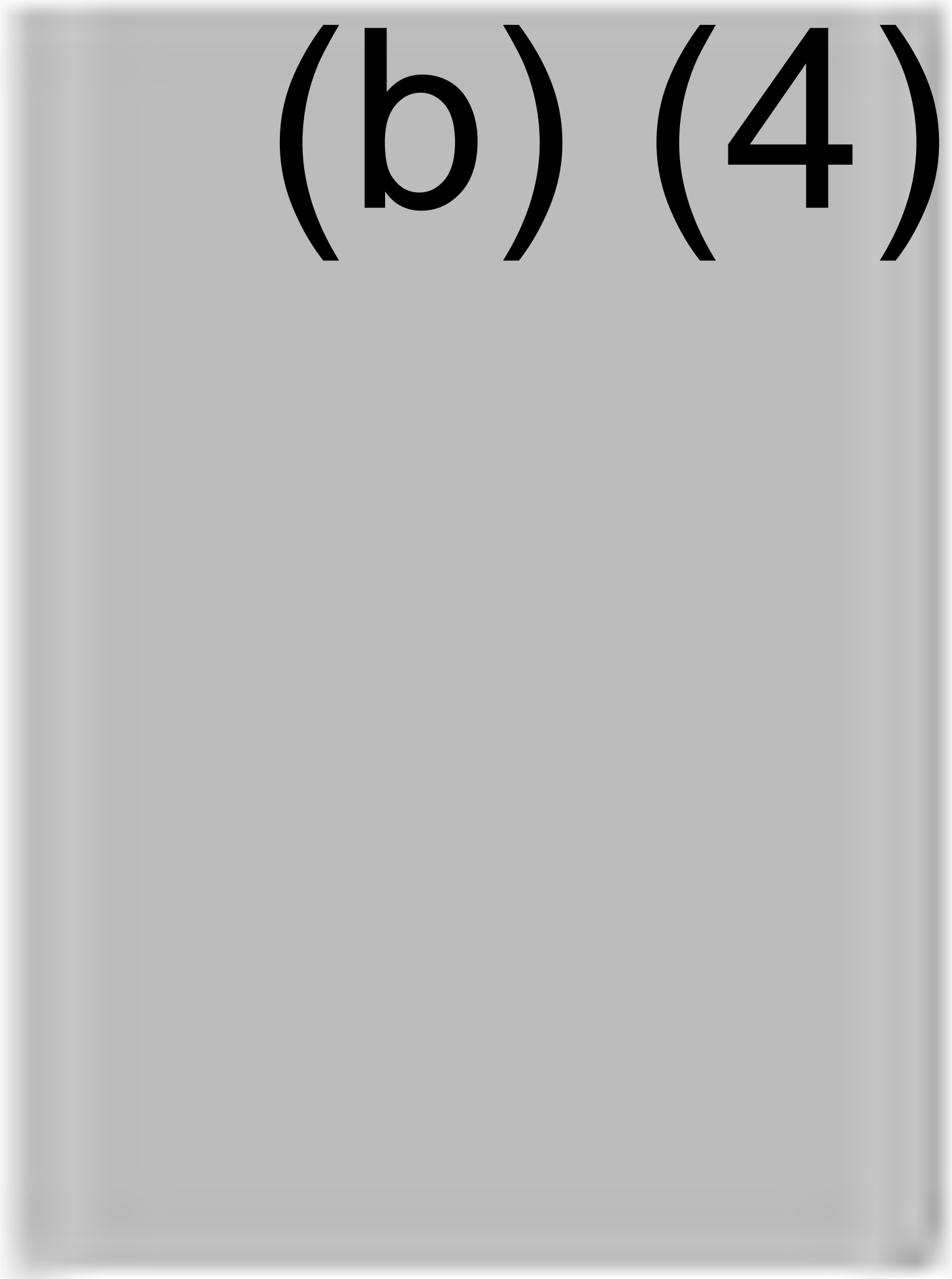

(b) (4)

AOAC Official Method 2015.01
Heavy Metals in Food
Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

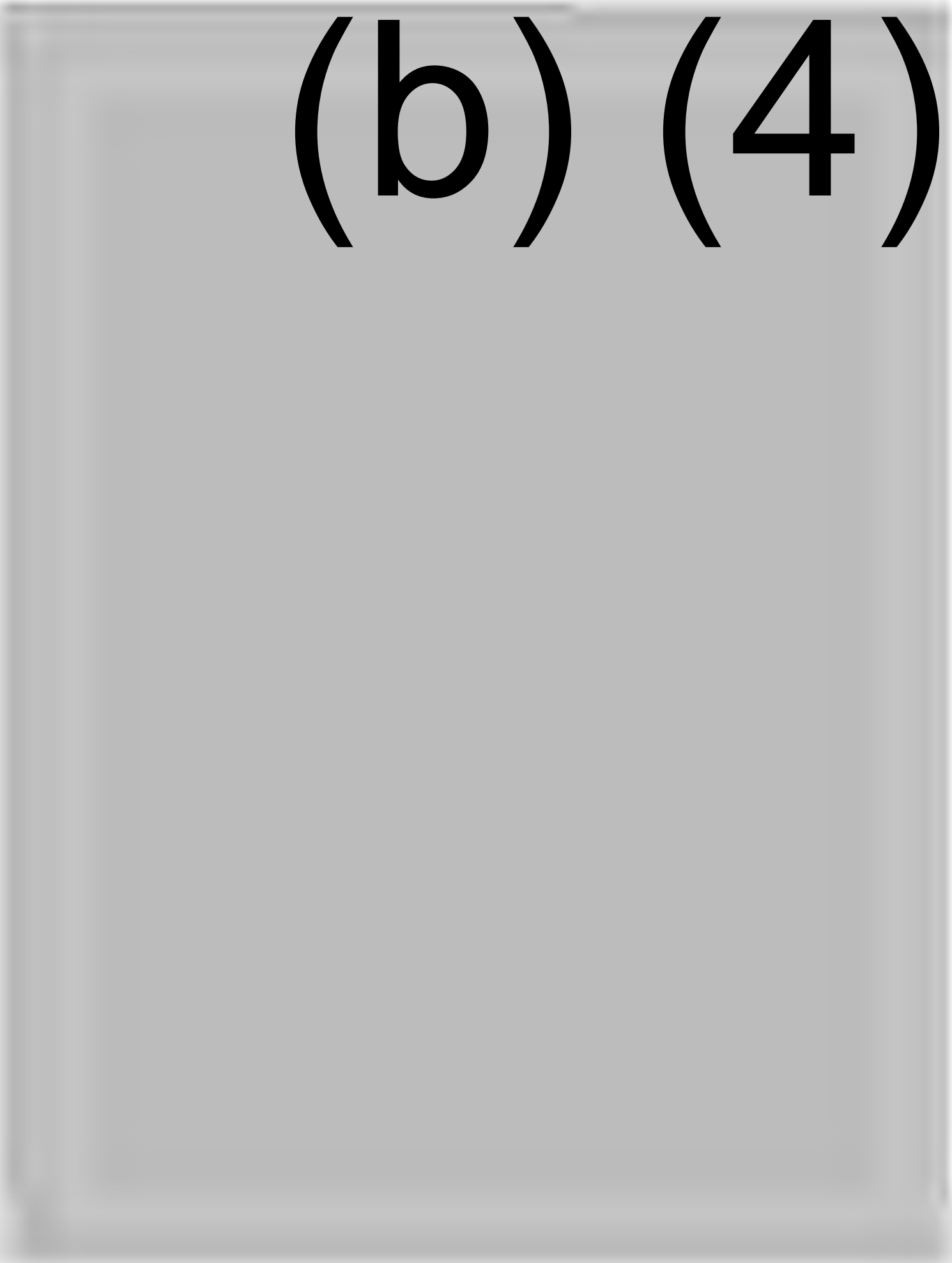
The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

(b) (4)

(b) (4)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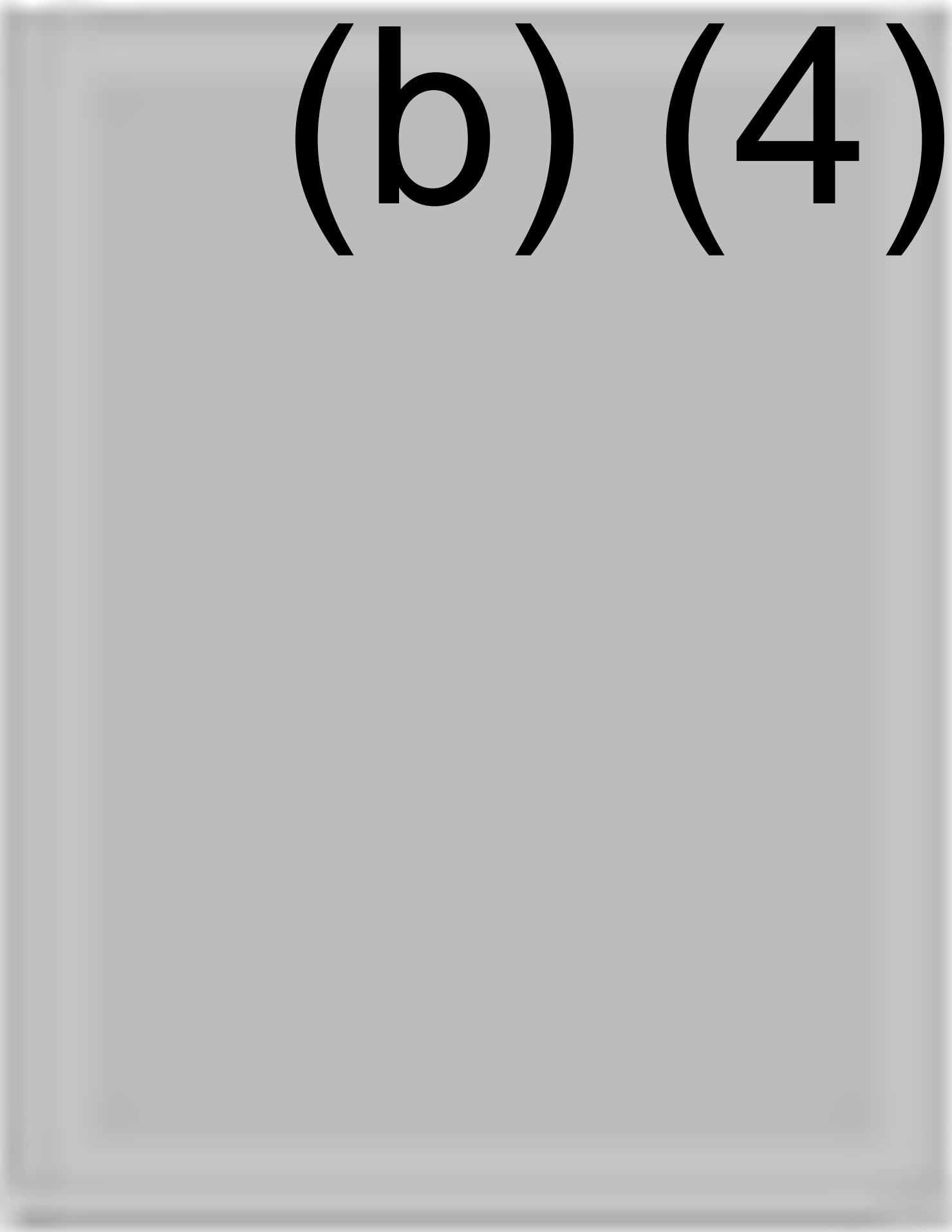
(b) (4)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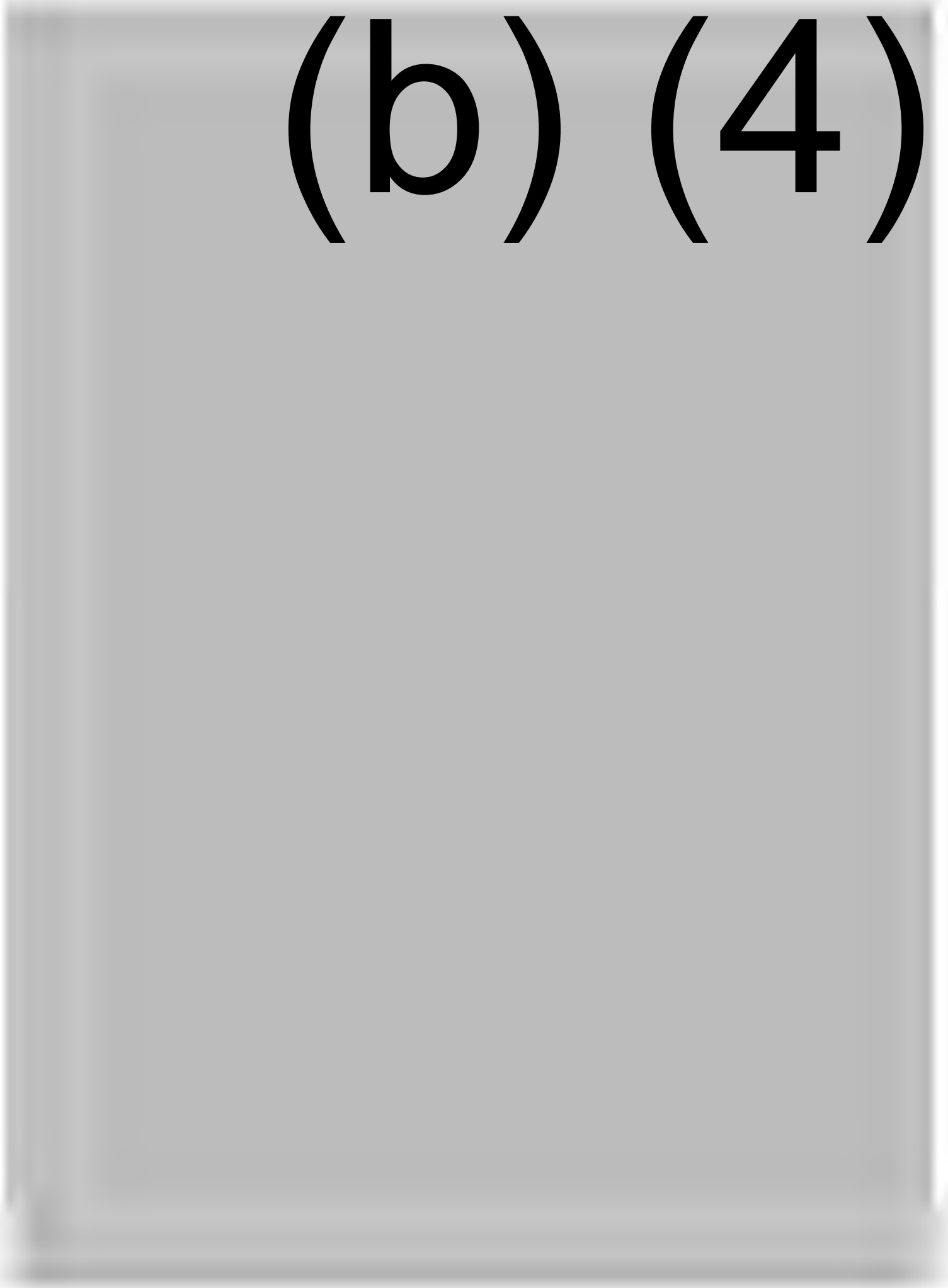
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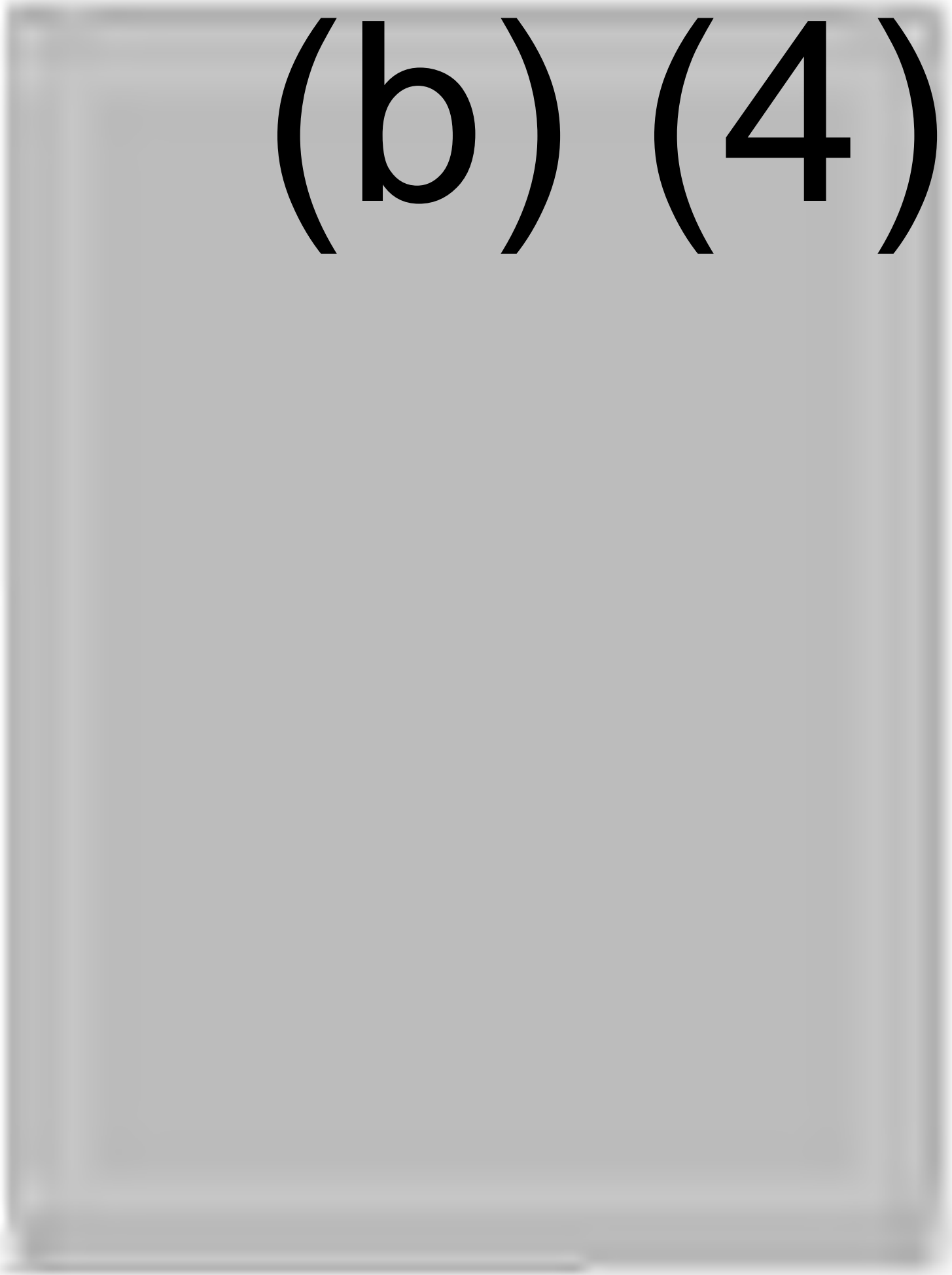
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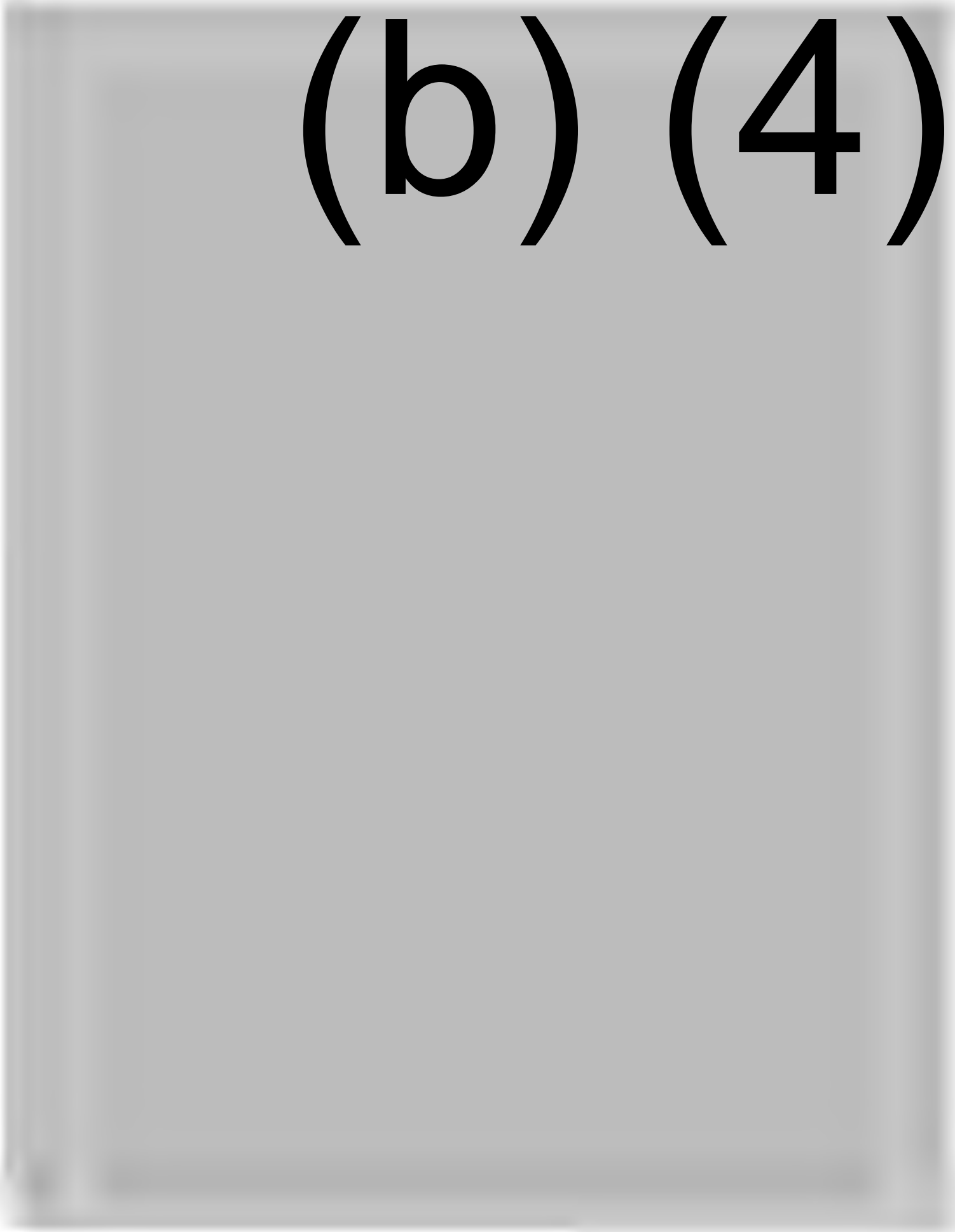
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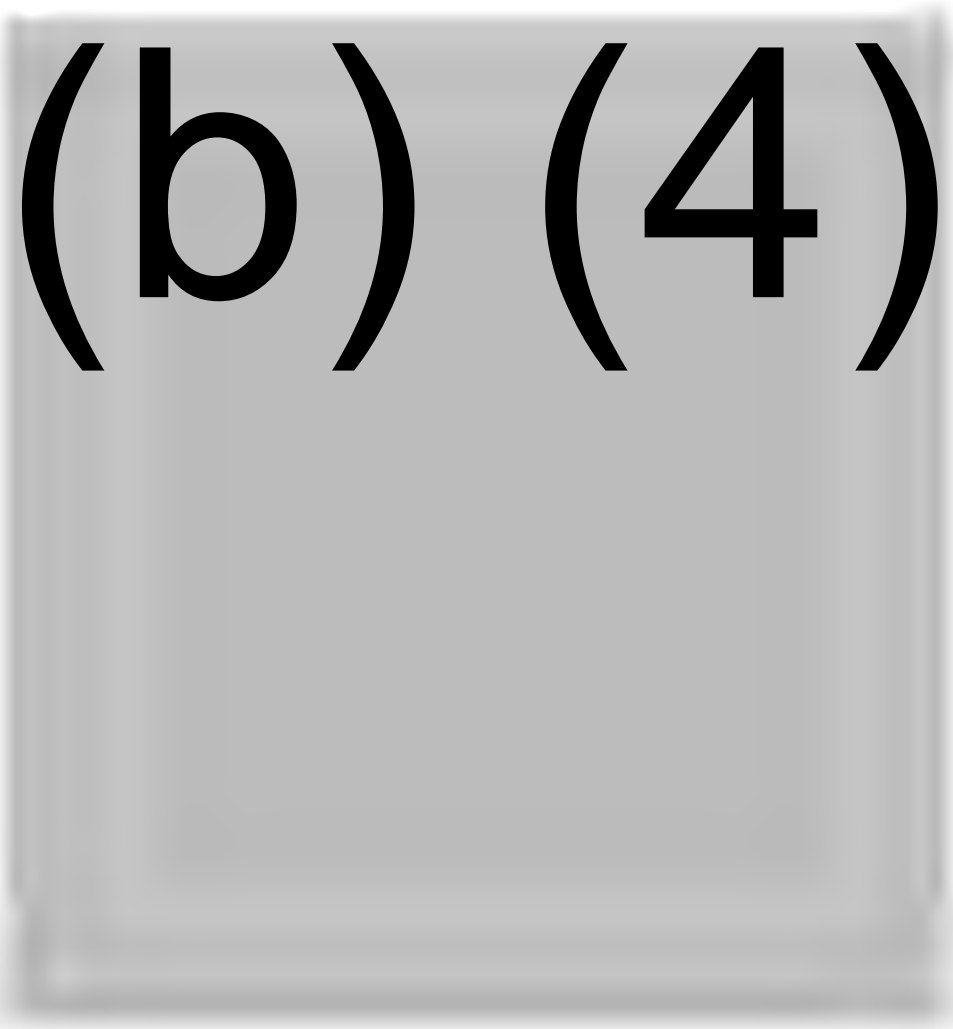


Method: **Determination of Heavy Metals by ICP-MS**

Reference: **AOAC Method 2015.01**

Approved: (b) (6)

Date: 4/25/19

1. Purpose

This method is to describe the steps for preparation of samples and standards to perform quantitative determination of metal impurities by microwave digestion and analysis by ICP-MS.

2. Scope

This method is applicable for the detection of metal impurities by ICP-MS. This method is suitable for a range of elements to be quantified; however, the elements of primary concern are arsenic, cadmium, lead and mercury.

3. Background

This method should be used by analysts familiar with trace element analysis and ICP-MS.

4. Responsibilities

4.1 Laboratory Co-Director authorized to assign and approve subject analysis is responsible for

- Approving Method Folder content
- Assuring the sample is fit for use
- Resolving analytical issues and deficiencies with subject analysis

4.2 Section Supervisor authorized to conduct subject analysis is responsible for

- Approving assigned analyst work
- Assuring the Method Folder is up to date including content and appendices
- Discussing any deviations with the Laboratory Co-Director

4.3 Analyst authorized to conduct this analysis is responsible for

- Reviewing Method Folder instructions prior to initiating analysis, especially for matrix applicability
- Analyzing the sample according to documented instructions
- Assessing method and instrument performance both real time and at reporting
- Addressing any deviation from instructions or specifications with the Section Supervisor
- Updating Method Folder performance data

5.0 References

5.1 Method

- AOAC INTERNATIONAL. Official Methods of Analysis, 20th ed., Method 2015.01 – Heavy Metals in Food – Inductively Coupled Plasma-Mass Spectrometry.
- FDA EAM (Elemental Analysis Manual) 4.7 Vesrion 1.1 (March 2015), P. Gray, W. Midak, J. Cheng – "Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic,

Cadmium, chromium, Lead, Mercury and Other Elements in Food Using Microwave Assisted Digestion"

- (b) (4)

5.2 Instrumentation

- (b) (4) 1000/2000 ICP-MS

6.0 Method Folder

6.1 Instrumentation

The analyst authorized to perform this test method must be deemed knowledgeable in the operation of the instrumentation cited in **5.2 Instrumentation**

6.2 Safety

This method does not address all safety issues associated with its use. The analyst must establish appropriate safety and health practice prior to initiating analysis. The analyst must be familiar with (b) (4) hazardous waste plan.

Reagents should be regarded as potential health hazards and exposure to these compounds should be limited.

6.3 Definitions

Analytical sample – sample, prepared by the laboratory (by homogenization, grinding, blending, etc.), from which analytical portions (aliquots) are removed for analysis.

Analytical portion – quantity of material removed from the analytical sample.

Analytical solution – solution prepared by decomposing an analytical portion and diluting to volume.

Batch – a group of analytical portions processed in a continuous sequence under relatively stable conditions. Specifically:

- Method is constant
- Instrument and its conditions (i.e. pertinent operating parameters) are constant
- Standardization is constant

Dilution Factor (DF) – factor by which concentration in a diluted solution (e.g. diluted analytical solution) is multiplied to obtain concentration in the initial solution (e.g. analytical solution).

Method Blank (MBK) – solution that is prepared using all reagents and exposed to all laboratory ware, apparatus, equipment, digestion process and analyses in the same manner as if it were an analytical portion being analyzed without the sample. The MBK is analyzed to ensure analytes have not significantly been added to the analytical portion from materials and laboratory environment.

Reagent Blank (RB) – solution that is prepared using the same labware, acids, and dilution as calibration standards, prepare a solution as if it were a calibration standard without added sample.

Reference material (RM) – food related materials developed for analytical quality control, which have reference value concentration for the element of interest.

Independent calibration verification (ICV) – solution of method analytes of known concentration obtained from a source external to the laboratory and different from the source used for instrument standardization. The ICV is used to ensure a valid standardization and to check laboratory performance.

Continuous calibration verification (CCV) – verification of one of the calibration standard points. It is used to verify the calibration accuracy during the analysis of the analytical batch.

Matrix Spike (SP) – analytical portion fortified (spiking) with the analyte before digestion. Measurement of the final concentration of the analyte is made according to the analytical method. The purpose of the spike is to determine if the preparation procedure or sample matrix contribute bias to the results.

Blank Spike (BS) – solution that is spiked with known concentration analytes and prepared using the same labware, acids, dilutions and exposed to the same digestion process as the Method Blank. The purpose is to determine the spiked analyte recoveries to determine the accuracy.

Internal Standards Solution (ISS) – non analyte solution that is added to all calibration standards, quality control and analyzed samples, which uses the isotope ratio to correct for the instrument drift and matrix interferences.

Stock standard solution – a solution containing a high concentration of the analyte purchased from a reputable commercial source. Stock standard solutions are used to prepare standard solutions and other needed analyte solutions.

Intermediate standard solution – a solution containing one or more analytes prepared in the laboratory by diluting an aliquot of stock solution.

Standard solution – a solution prepared from the dilution of stock standard or intermediate standard solutions. Standard solutions are used to standardize instrument response (absorbance) to analyte concentration.

Analytical solution detection limit (ASDL) – an estimate of the lowest concentration of the analyte element in a MBK according to the statistics of hypothesis with a 95% confidence.

Limit of detection (LOD) – an estimate of the element concentration a method can detect in an analytical portion according to the statistics of hypothesis testing with a 95% confidence.

Limit of Quantitation (LOQ) – the minimum concentration of an analyte in a specific matrix that can be reliably quantified while also meeting predefined goals for bias and imprecision.

7.0 Method Work Level Instructions

7.1 Equipment and materials

(a) Analytical Balance – capable of weighing to the nearest 0.001 gram.
(b) Digestion vials – disposable glass tubes
(c) Microwave Digestor – (b) (4)
(d) ICP-MS – (b) (4)

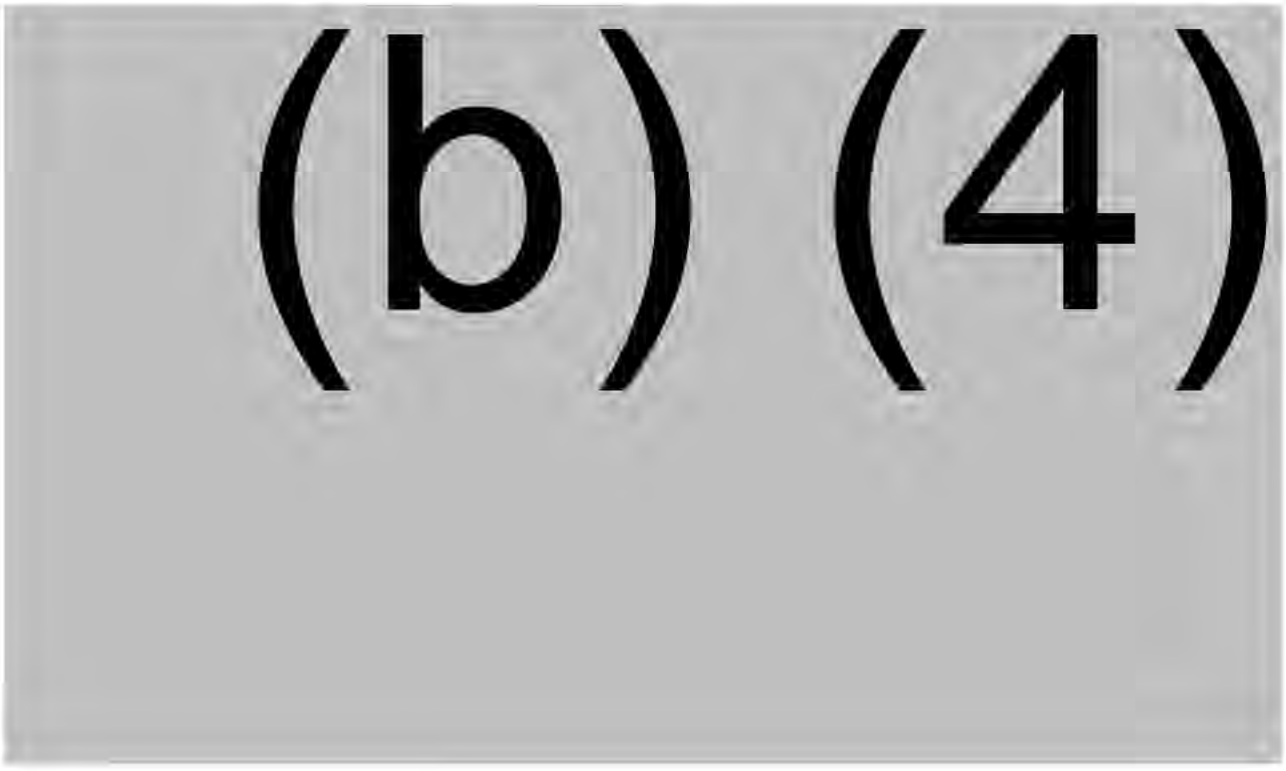
7.2 Reagents and Standards

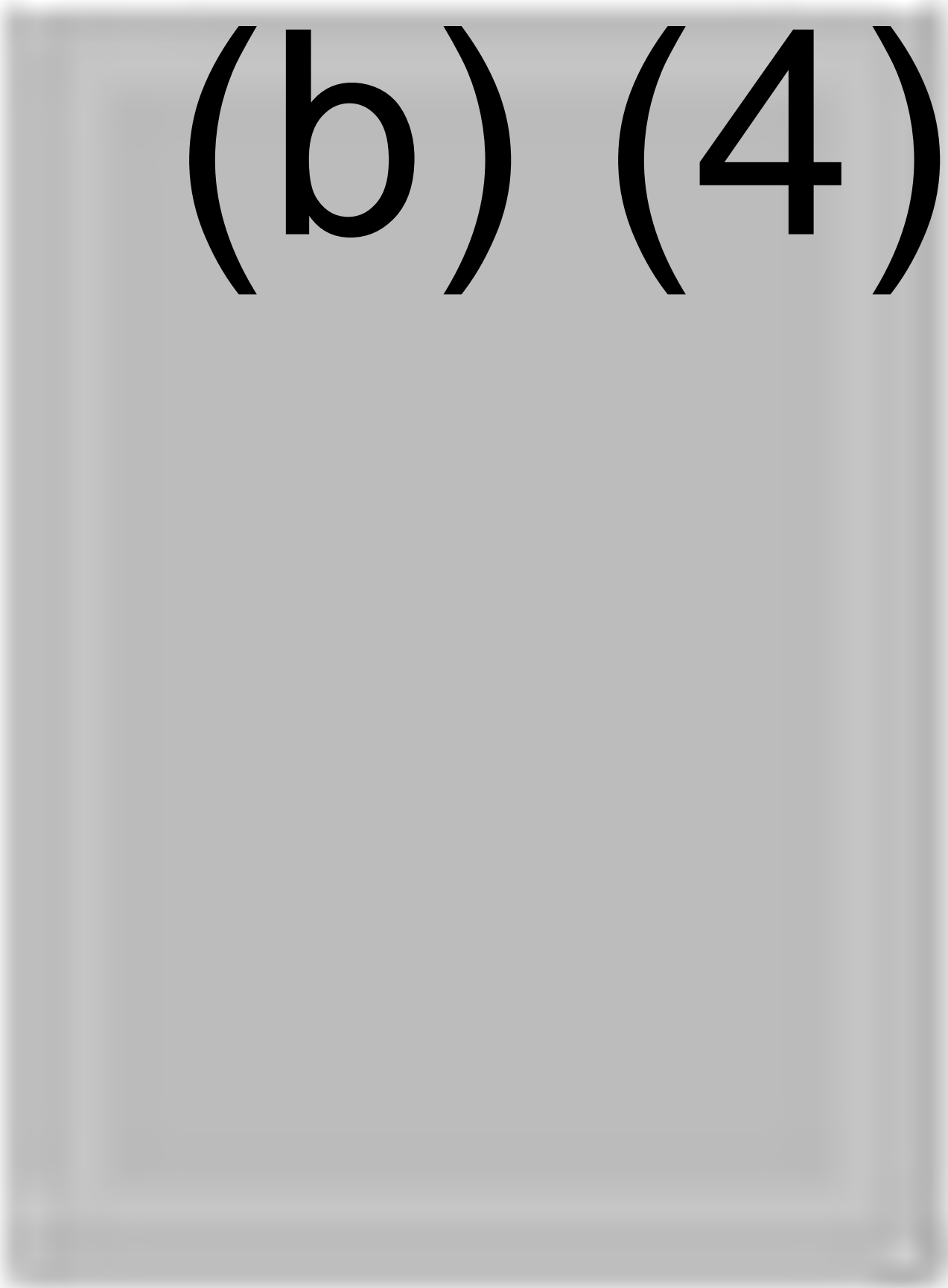
All reagents may contain impurities that may affect the integrity of the analytical results. Due to the high sensitivity of the ICP-MS, high-purity reagents, water, acids, glassware and sample tubes that are suitable for trace metal analysis must be used at all time.

(a) (b) (4) mg/L (ppm) Gold (Au) Stock Standard
(b) mg/L (ppm) Arsenic (As) Stock Standard
(c) mg/L (ppm) Cadmium (Cd) Stock Standard
(d) mg/L (ppm) Lead (Pb) Stock Standard
(e) mg/L (ppm) Mercury (Hg) Stock Standard
(f) Nitric Acid (HNO_3) – Concentrated (sp gr 1.41), trace metal grade
(g) Hydrochloric Acid (HCl) – Concentrated, trace element grade
(h) Internal Standard Solution – (b) (4) mg/L Germanium (Ge), (b) (6), (mg/L Gallium (Ga), (b) mg/L Indium (In), (b) mg/L Terbium (Tb)
(i) Deionized water (DI H_2O)

7.2.1 Working solutions

Please always use safety precautions when preparing solutions. Always add acid to water! Shake each solution after all the reagents are combined.




(b) (4)

7.3 Test Sample Treatment

(b) (4) microwave is used to digest in order to prepare the analytical batch.

7.3.1 Sample Preparation:

(b) (4)

(b) (4)

7.4 Instrumentation Set up

(b) (4)

7.4.3 Running Samples:

(b) (4)

(b) (4)

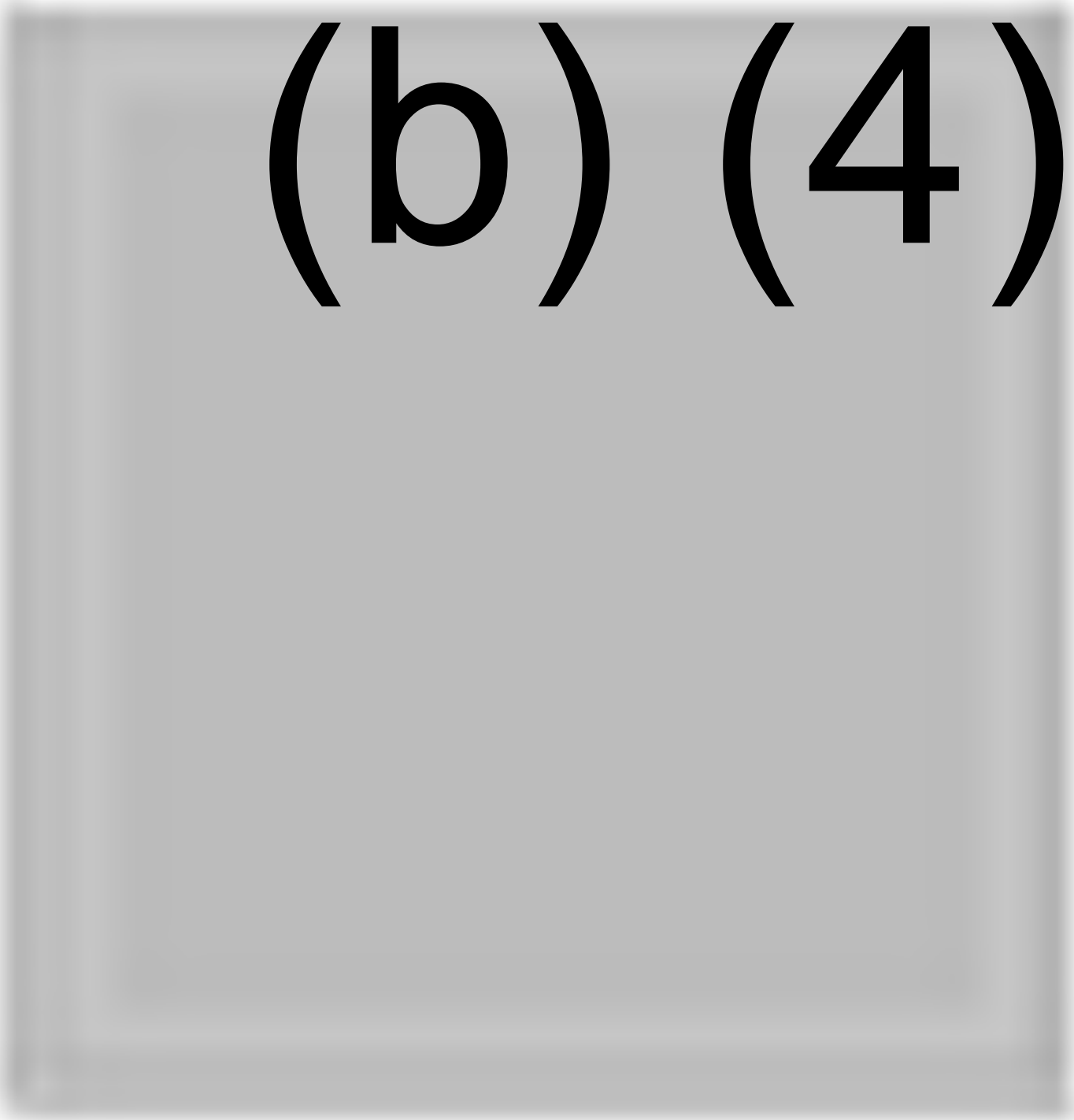
7.4.4 While Running:

(b) (4)

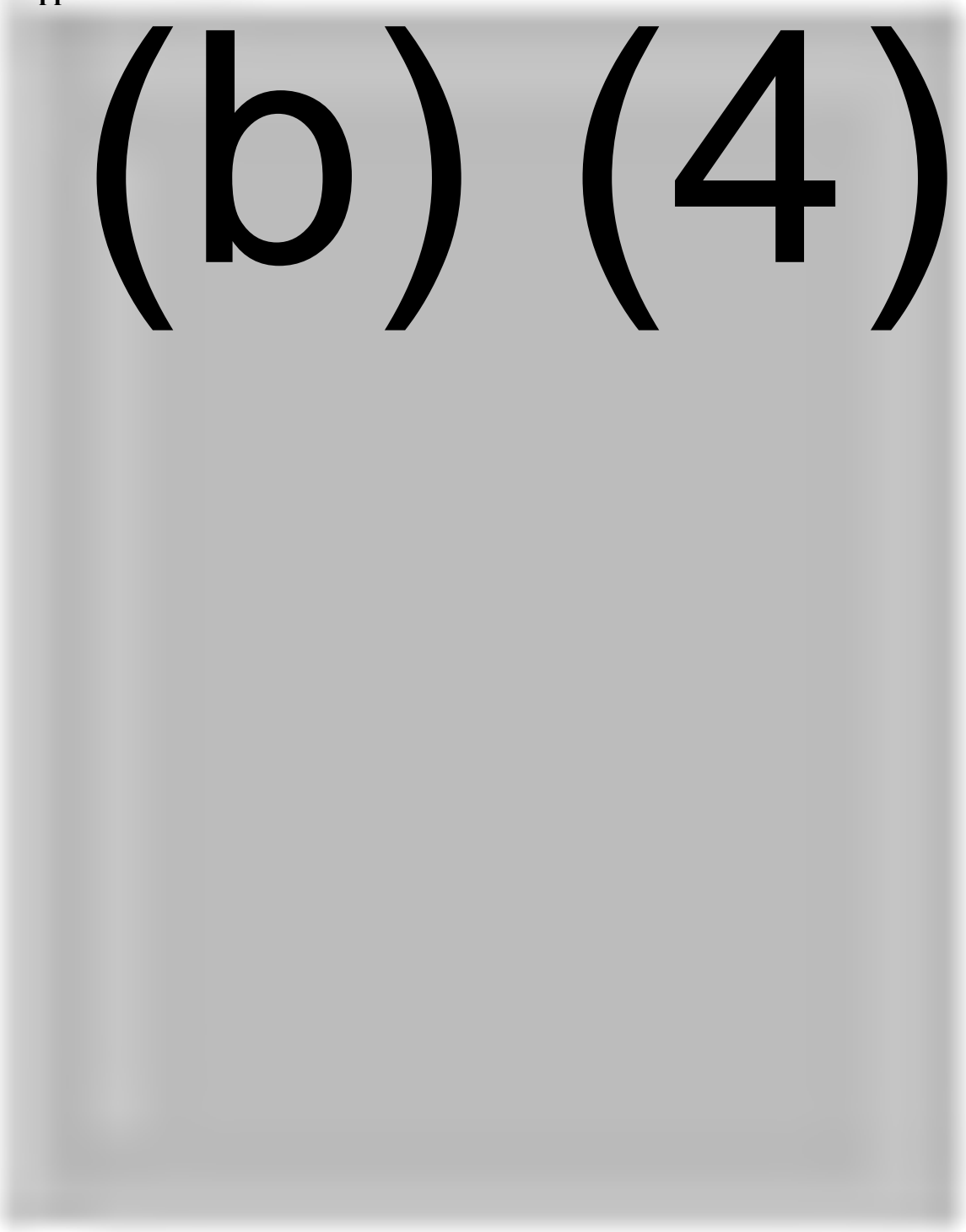
7.4.5 Data Processing:

(b) (4)

Appendix A - Calibration Concentrations



Appendix B - Solutions Guide



AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

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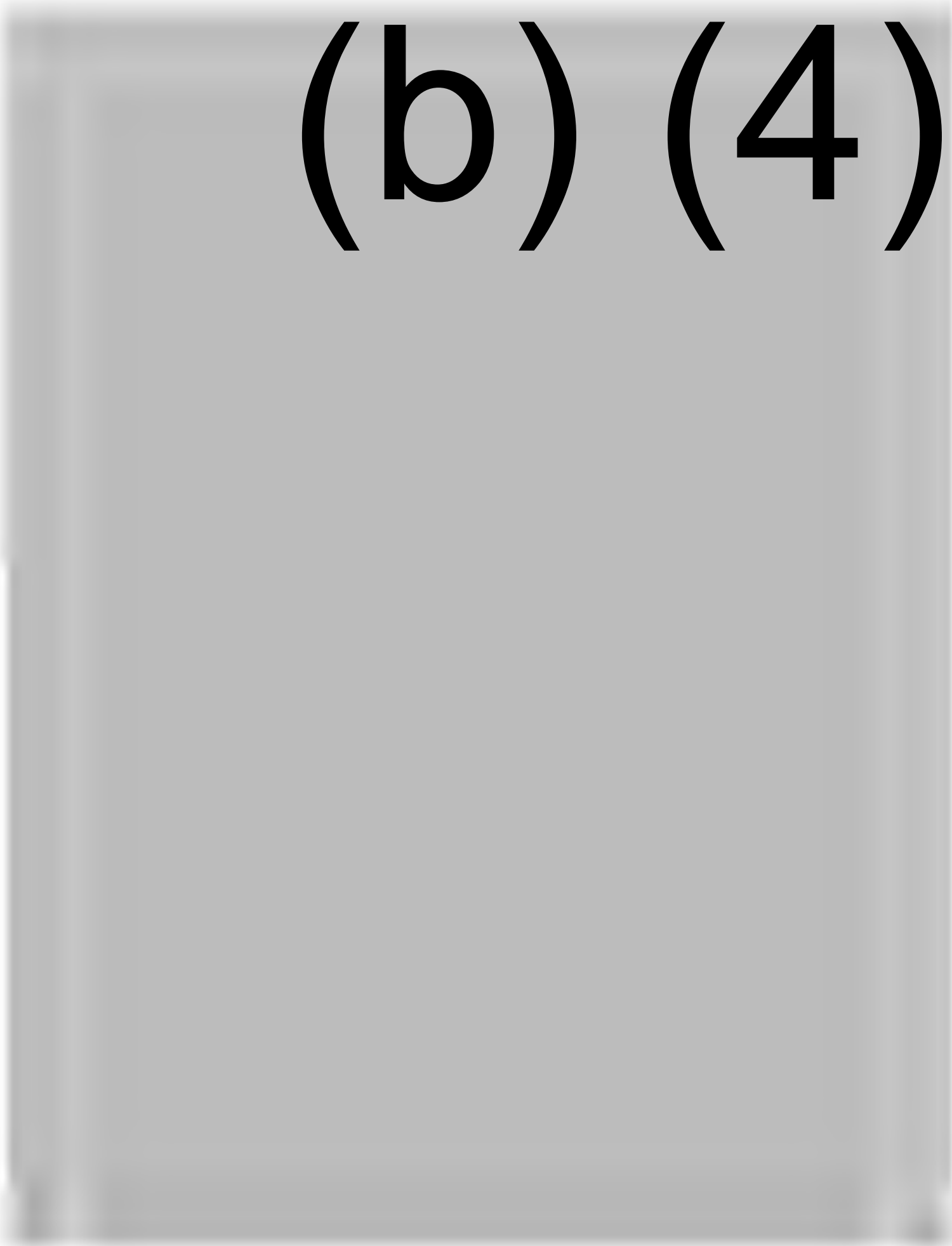
(b) (4)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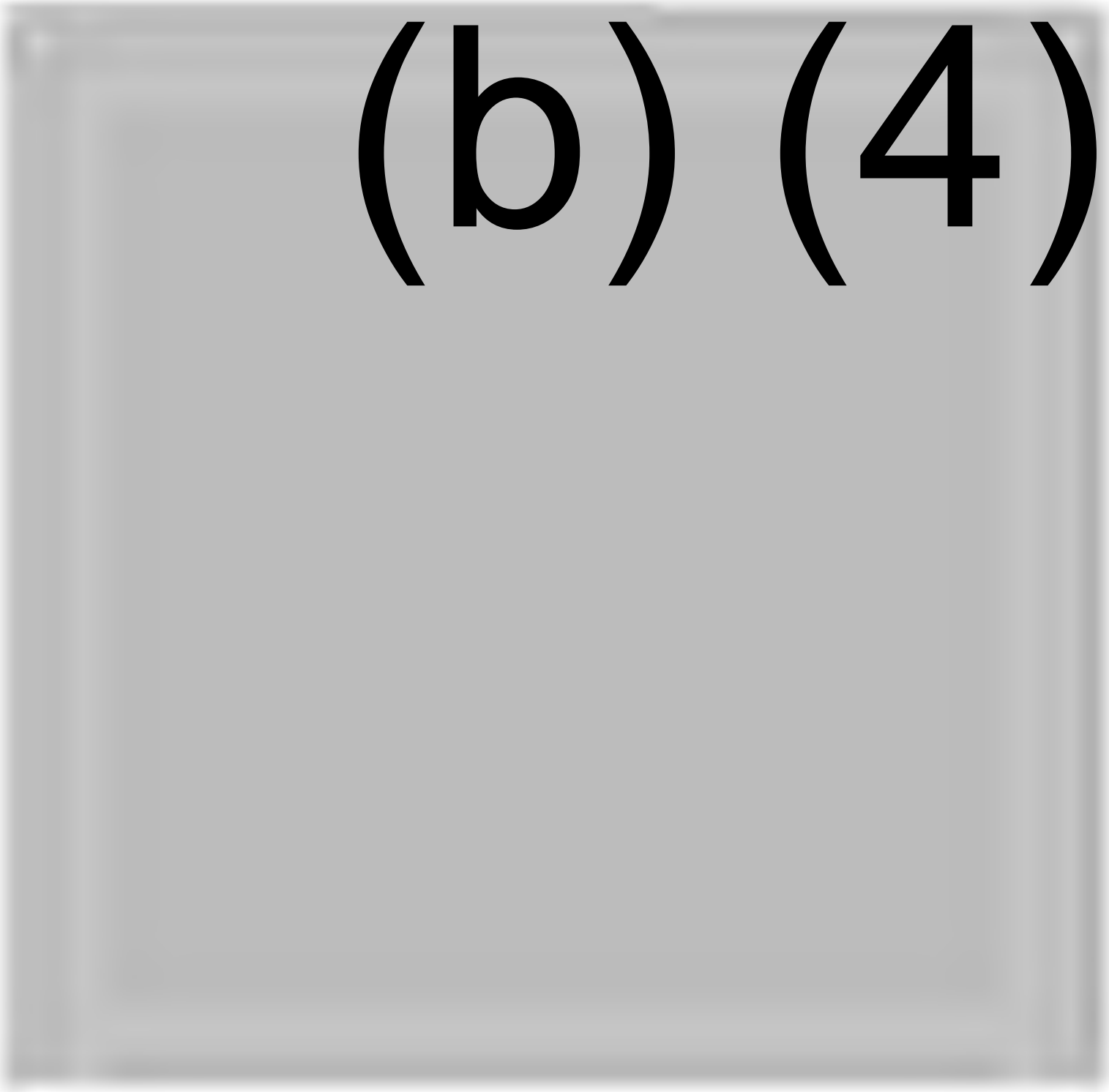


AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

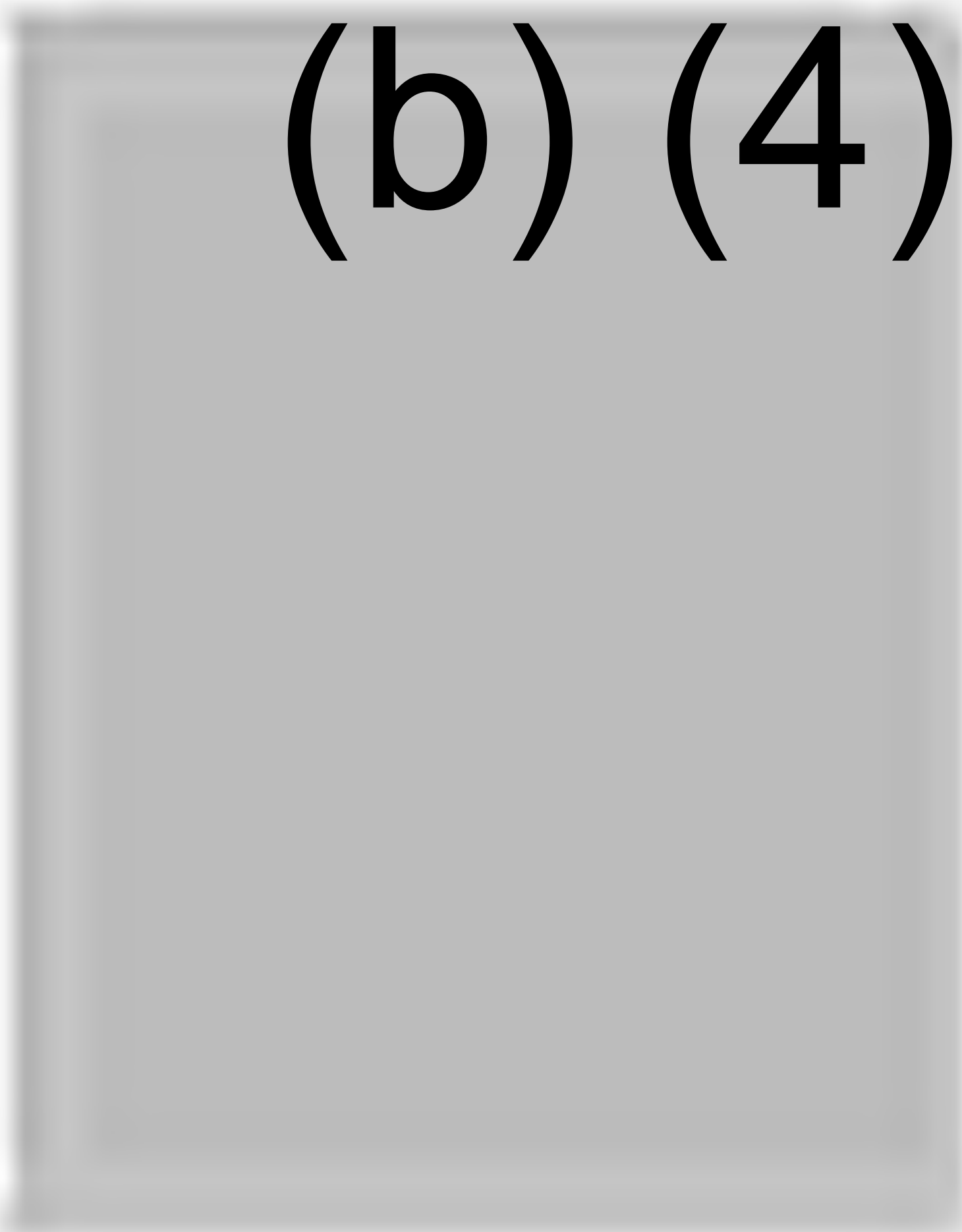
See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

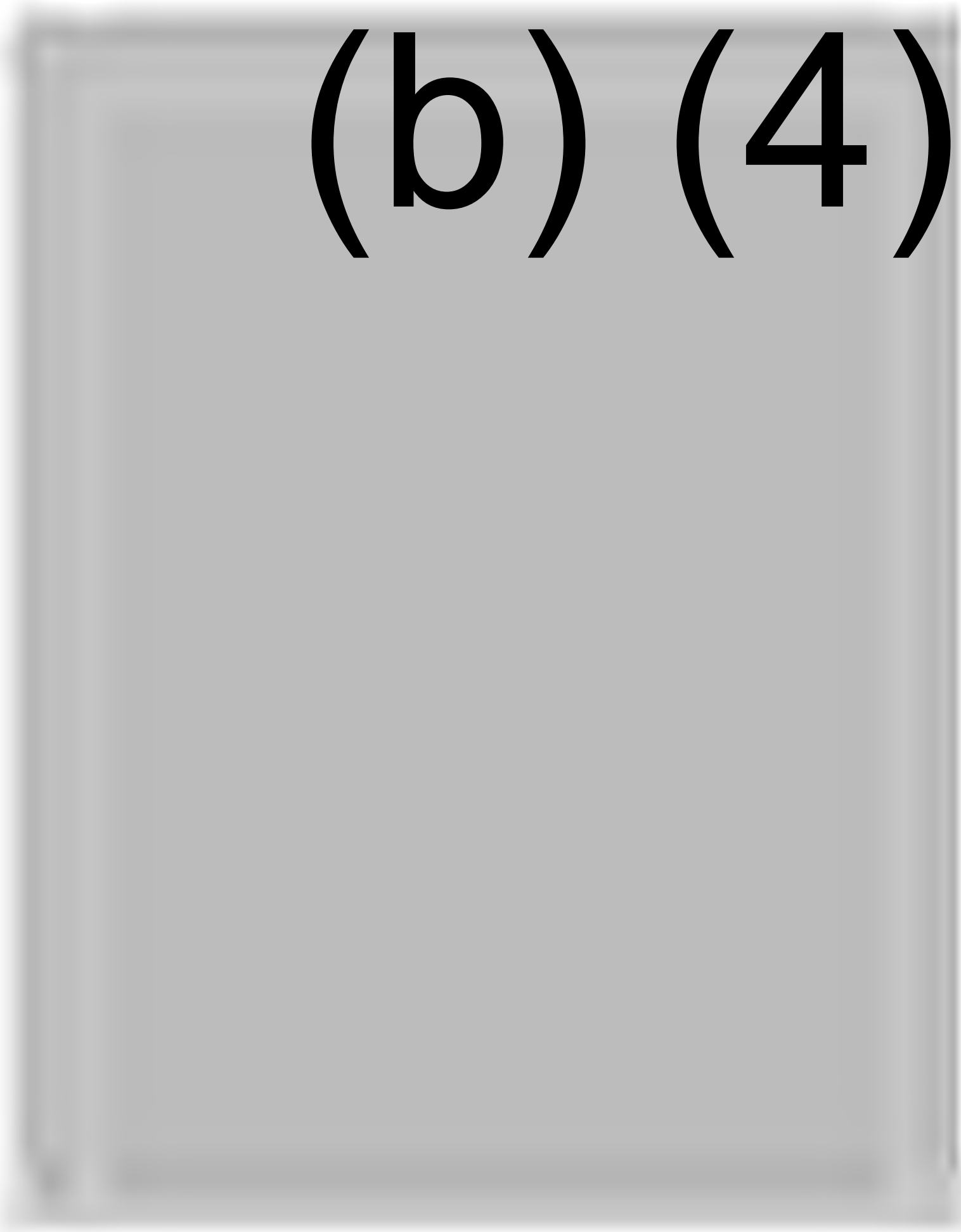
Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

(b) (4)

(b) (4)

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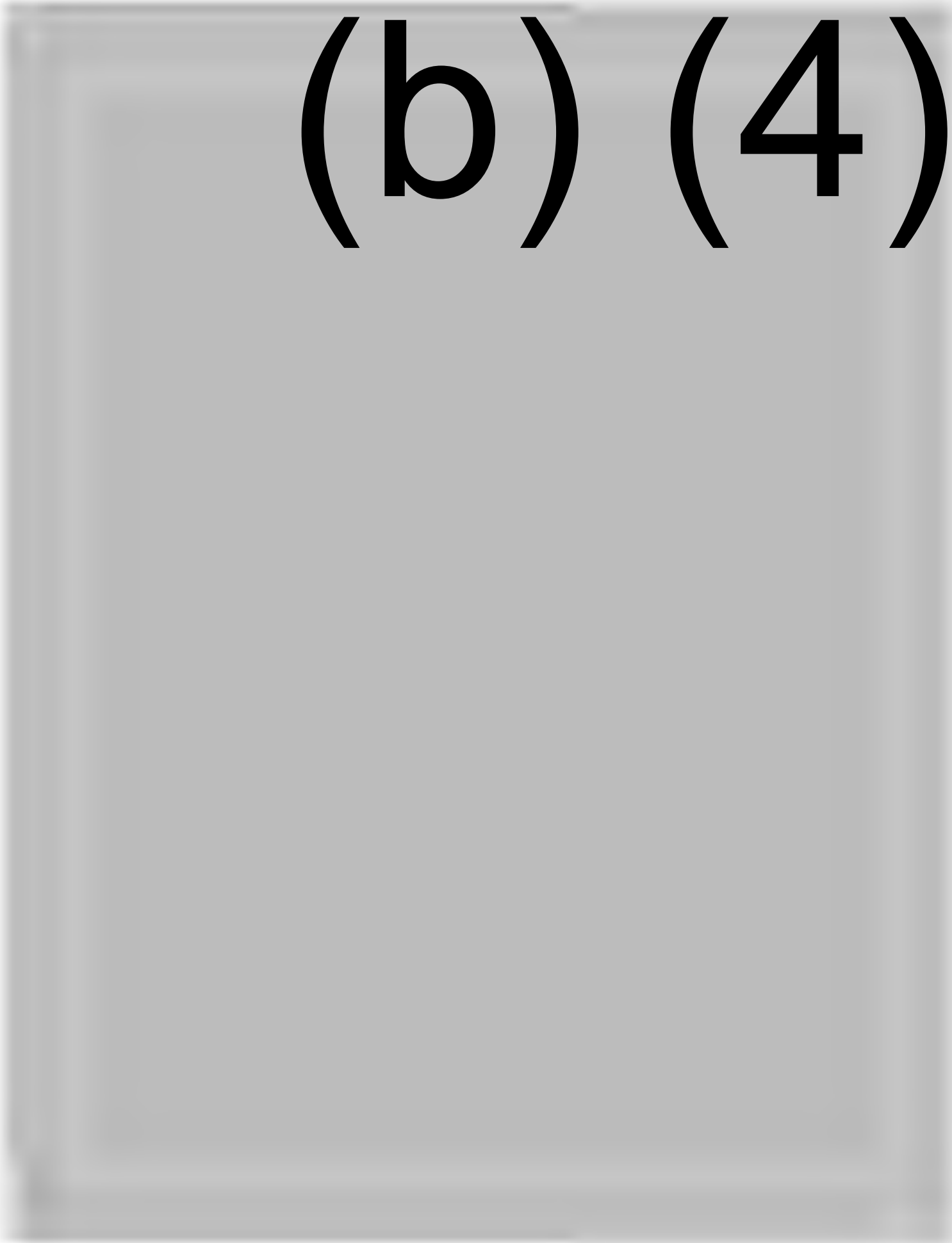
(b) (4)

Appendix 008

[redacted] (b) (4)

[redacted] (b) (6), (b) (4)

Name: Ascus Biosciences Inc.
Customer: Martin Mayhew
Address: 6450 Lusk Blvd. Suites E109/209
San Diego, CA
92121
USA
877-696-8945

Order ID: [redacted] (b) (4)
Report ID:
Date Received: 12/11/2020 11:17:20
Reported: 12/16/2020 16:24:21
P.O. #: N/A
Page: 1 of 1

Report of Results

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4) **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: [redacted] (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] (b) (4), (b) (6)

(b) (6), (b) (4)

CERTIFICATE OF ANALYSIS

Customer: (b) (4)

Product: Ammonium Hydroxide 29% **Sales Order #:** (b) (4)

Purchase Order #: (b) (4) **Shipment Date:** 6/24/2019

Lot #: 05-02-19-01

	Analysis
Ammonia, wt.%:	29.9
Specific Gravity @ 60°F, g/mL:	0.896
Appearance:	Clear, Colorless

(b) (4)

This document was produced electronically and no signature is required.

(b) (4)

Specification for Ascorbic Acid, USP (b) (4)

Item Number	(b) (4)
Item	Ascorbic Acid, USP
CAS Number	50-81-7
Molecular Formula	$C_6H_8O_6$
Molecular Weight	176.13
MDL Number	
Synonyms	Vitamin C ; L-Ascorbic Acid

Test	Specification	
	Min	Max
APPEARANCE		
IDENTIFICATION A (FTIR)	(b) (4) MATCHES REFERENCE	
IDENTIFICATION (B)	**REDUCES ALKALINE CUPRIC TARTRATE TS**	
ASSAY	**99.0**	**100.5 %**
SPECIFIC ROTATION $[a]_D$	+20.5 to+21.5	
RESIDUE ON IGNITION		0.1 %
ELEMENTAL IMPURITIES:		
LEAD (Pb)		**AS REPORTED**
ARSENIC (As)		**AS REPORTED**
THALLIUM (Tl)		AS REPORTED
GOLD (Au)		AS REPORTED
SELENIUM (Se)		AS REPORTED
CERTIFIED KOSHER		
CERTIFIED HALAL		
EXPIRATION DATE		
DATE OF MANUFACTURE		
RESIDUAL SOLVENTS		AS REPORTED

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Ascorbic Acid, USP	CAS Number	50-81-7
Molecular Formula	$C_6H_8O_6$	Molecular Weight	176.13

TEST	SPECIFICATION		RESULT
	MIN	MAX	
APPEARANCE			WHITE CRYSTALS
IDENTIFICATION A (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION (B)	REDUCES ALKALINE CUPRIC TARTRATE TS		REDUCES ALKALINE CUPRIC TARTRATE TS
ASSAY	99.0	100.5 %	99.7 %
SPECIFIC ROTATION $[a]_D$	+20.5 to+21.5		+21.0
RESIDUE ON IGNITION		0.1 %	0.04 %
ELEMENTAL IMPURITIES:			.
LEAD (Pb)		AS REPORTED	<0.5 ppm
ARSENIC (As)		AS REPORTED	<1.5 ppm
THALLIUM (Tl)		AS REPORTED	<0.8 ppm
GOLD (Au)		AS REPORTED	<10 ppm
SELENIUM (Se)		AS REPORTED	<8 ppm
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			14-MAR-2025
DATE OF MANUFACTURE			18-MAR-2022
RESIDUAL SOLVENTS		AS REPORTED	.
CLASS 2 (SOLVENT) / METHANOL			<3000 ppm

(b) (4)

Specification for Biotin, Powder, FCC (b) (4)

Item Number	(b) (4)
Item	Biotin, Powder, FCC
CAS Number	58-85-5
Molecular Formula	$C_{10}H_{16}N_2O_3S$
Molecular Weight	244.31
MDL Number	
Synonyms	Vitamin H

Test	Specification	
	Min	Max
ASSAY ($C_{10}H_{16}N_2O_3S$)	97.5	100.5 %
MELTING RANGE	229°	232°C (dec.)
OPTICAL ROTATION, [a] 20D	+89° to+93°	
LEAD (Pb)		2 mg/kg
IDENTIFICATION		TO PASS TEST
CERTIFIED KOSHER		
CERTIFIED HALAL		
APPEARANCE		
RETEST DATE		
DATE OF MANUFACTURE		

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Biotin, Powder, FCC	CAS Number	58-85-5
Molecular Formula	$C_{10}H_{16}N_2O_3S$	Molecular Weight	244.31

TEST	SPECIFICATION		RESULT
	MIN	MAX	
ASSAY ($C_{10}H_{16}N_2O_3S$)	97.5	100.5 %	99.5 %
MELTING RANGE	229°	232°C (dec.)	231.5 - 231.9 °C
OPTICAL ROTATION, [a] 20D	+89° to+93°		+90.7°
LEAD (Pb)		2 mg/kg	<0.5 mg/kg
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE POWDER
RETEST DATE			15-MAY-2027
DATE OF MANUFACTURE			16-MAY-2023
MONOGRAPH EDITION			FCC 13

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Calcium Chloride, Anhydrous, Granular, FCC		
CAS Number	10043-52-4		
Molecular Formula	$CaCl_2$	Molecular Weight	110.98

Test	Specification		Result
	min	max	
ASSAY ($CaCl_2$)	93.0 - 100.5 %		96.3 %
ACID-INSOLUBLE MATTER		TO PASS TEST	PASSES TEST
ARSENIC (As)		3 mg/kg	<3 mg/kg
FLUORIDE		0.004 %	0.001 %
LEAD (Pb)		5 mg/kg	<5 mg/kg
MAGNESIUM AND ALKALI SALTS		5.0 %	2.7 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
pH of a 35.5 % Solution	7.0		9.6
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
RETEST DATE			31-MAY-2022
DATE OF MANUFACTURE			01-JUN-2017
APPEARANCE			WHITE GRANULES

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Calcium Pantothenate, Powder, USP		
CAS Number	137-08-6		
Molecular Formula	$C_{18}H_{32}CaN_2O_{10}$	Molecular Weight	476.53

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.0	102.0 %	98.1 %
CALCIUM CONTENT (Ca; DRIED BASIS)	8.2	8.6 %	8.36 %
OPTICAL ROTATION	+25.0° to +27.5°		+26.0°
LOSS ON DRYING		5.0 %	3.0 %
ALKALINITY	NO PINK COLOR		NO PINK COLOR
ELEMENTAL IMPURITIES:			.
CADMIUM (Cd)	AS REPORTED		<1 µg/g
LEAD (Pb)	AS REPORTED		<2 µg/g
ARSENIC (As)	AS REPORTED		<1 µg/g
MERCURY (Hg)	AS REPORTED		<0.1 µg/g
CHROMIUM (Cr)	AS REPORTED		<0.1 µg/g
IDENTIFICATION (A)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION (B)	POSITIVE FOR CALCIUM		POSITIVE FOR CALCIUM
IDENTIFICATION (C)	+25.0°to+27.5°		+26.0°
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			31-MAY-2022
DATE OF MANUFACTURE			01-JUN-2019
APPEARANCE			WHITE POWDER
RESIDUAL SOLVENTS	AS REPORTED		.
CLASS 2 (SOLVENT) / METHANOL			<3000 ppm

(b) (4), (b) (6)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Copper Sulfate, Pentahydrate, Granular, FCC		
CAS Number	7758-99-8		
Molecular Formula	$CuSO_4.5H_2O$	Molecular Weight	249.69

Test	Specification		Result
	min	max	
ASSAY ($CuSO_4.5H_2O$)	98.0 - 102.0 %		99.33 %
IRON (Fe)		0.01 %	0.001 %
LEAD (Pb)		4 mg/kg	2 mg/kg
SUBSTANCES NOT Pptd.BY HYDROGEN SULFIDE		0.3 %	0.18 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
EXPIRATION DATE			31-JAN-2018
MANUFACTURE DATE			27-DEC-2013
APPEARANCE			BLUE GRANULES

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Dibasic Potassium Phosphate, Anhydrous, USP, EP, BP	Manufacturer Lot	(b) (4)
CAS Number	7758-11-4	Manufacturer Code	(b) (4)
Molecular Formula	K_2HPO_4	Molecular Weight	174.18

Test	Specification		Result
	min	max	
ASSAY (K_2HPO_4; DRIED BASIS)	98.0	100.5 %	99.3 %
pH OF A 1 IN 20 SOLUTION	8.5	9.6	8.9
LOSS ON DRYING		1.0 %	0.15 %
INSOLUBLE SUBSTANCES		0.2 %	0.00 %
CARBONATE	NOT MORE THAN A FEW BUBBLES ARE EVOLVED		NOT MORE THAN A FEW BUBBLES ARE EVOLVED
CHLORIDE (Cl)		0.02 %	<0.02 %
SULFATE		0.1 %	<0.1 %
ARSENIC (As)		2 ppm	0.2 ppm
IRON (Fe)		0.001 %	<0.001 %
SODIUM (Na)	NO YELLOW COLOR		NO YELLOW COLOR
SODIUM (EP)		0.1 %	<0.05 %
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
FLUORIDE		0.001 %	0.0003 %
MONOBASIC OR TRIBASIC SALT		0.4 ml	<0.4 ml
MONOPOTASSIUM PHOSPHATE		2.5 %	1.1 %
APPEARANCE OF SOLUTION	CLEAR AND COLORLESS		CLEAR AND COLORLESS
REDUCING SUBSTANCES	SOLUTION REMAINS PINK		SOLUTION REMAINS PINK
IDENTIFICATION (A)	POSITIVE FOR POTASSIUM		POSITIVE FOR POTASSIUM
IDENTIFICATION (B)	POSITIVE FOR PHOSPHATE		POSITIVE FOR PHOSPHATE
IDENTIFICATION (C)	SLIIGTHLY ALKALINE		SLIIGTHLY ALKALINE
CERTIFIED KOSHER			CERTIFIED KOSHER
APPEARANCE			WHITE POWDER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			04-MAR-2023
DATE OF MANUFACTURE			04-MAR-2020
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION (USP)			(USP) 42
MONOGRAPH EDITION (EP)			(EP) 10
MONOGRAPH EDITION (BP)			(BP) 2020


(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Folic Acid, Powder, USP		
CAS Number	59-30-3		
Molecular Formula	$C_{19}H_{19}N_7O_6$	Molecular Weight	441.40

Test	Specification		Result
	min	max	
ASSAY (ANHYDROUS BASIS)	97.0	102.0 %	97.9 %
WATER DETERMINATION		8.5 %	8.2 %
RESIDUE ON IGNITION		0.3 %	0.12 %
RELATED COMPOUNDS		2.0 %	< 2.0 %
ELEMENTAL IMPURITIES:			.
CADMIUM (Cd)	AS REPORTED		< 1 ug/g
LEAD (Pb)	AS REPORTED		< 0.5 ug/g
ARSENIC (As)	AS REPORTED		< 1 ug/g
MERCURY (Hg)	AS REPORTED		< 0.1 ug/g
IDENTIFICATION A . ULTRAVIOLET ABSORPTION	The ratio A256 / A365 is 2.80 - 3.00		2.88
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			26-SEP-2021
DATE OF MANUFACTURE			27-SEP-2019
APPEARANCE			ORANGE POWDER
RESIDUAL SOLVENTS:	AS REPORTED		NO RESIDUAL SOLVENTS USED

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Hydrochloric Acid, 37 Percent, FCC		
CAS Number	7647-01-0		
Molecular Formula	HCl	Molecular Weight	36.46

Test	Specification		Result
	min	max	
ASSAY	36.0 - 38.0 %		37.5 %
COLOR		TO PASS TEST	PASSES TEST
SPECIFIC GRAVITY		TO PASS TEST	PASSES TEST
IRON (Fe)		5 mg/kg	<0.01 mg/kg
LEAD (Pb)		1 mg/kg	<0.01 mg/kg
MERCURY		0.10 mg/kg	<0.001 mg/kg
NONVOLATILE RESIDUE		0.5 %	<0.0005 %
ORGANIC COMPOUNDS		TO PASS TEST	PASSES TEST
OXIDIZING SUBSTANCES (as Cl2)		0.003 %	<0.0001 %
REDUCING SUBSTANCES (as SO_3)		0.007 %	<0.007 %
SULFATE		0.5 %	<0.00001 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
EXPIRATION DATE			28-FEB-2021
DATE OF MANUFACTURE			28-FEB-2019
APPEARANCE			CLEAR COLORLESS LIQUID

(b) (6), (b) (4)

27 Stearine

(b) (4)

27 Stearine™

(b) (4)

Product Data Sheet

Product Description:
27 Stearine is a highly functional hardened palm oil. Palm stearines crystallize into a stable betaprime configuration. Beta-prime hard fats crystallize into permanent fine grained crystals. This allows for maximum oil stabilization as well as stability over a broad range of storage conditions.

Typical data suggests that it may be used for stabilizing peanut butter, as well as a melt point adjuster for many types of processed foods. The user is advised to fully evaluate the functionality and shelf life of the shortening in their intended finished product at their own facilities, as performance may be affected by varying formulations and process conditions.

Ingredient Statement:
Hydrogenated Palm Oil, Kosher, (US)

Typical Data:

Capillary Melting Point	.....58-62°C/136-144°F
Color (5 1/4") Lovibond	.....5 R max
Free Fatty Acid (% as oleic)	.....0.10% max
Iodine Value	.....4 max

The typical data provided here is valid at the point of shipment from our manufacturing facility

Packaging:
27 Stearine is available in 50 lb. beaded poly-lined cartons and in bulk liquid.

Storage and Handling:
27 Stearine needs no refrigeration, however, like all fats, it will absorb odors and should be stored between 40-80°F in a dry place away from odor-producing substances. Bulk liquid product can be stored at 150-160°F for 30 days. Based on the typical data a shelf-life of 180 days is suggested for packaged product stored at 40-80°F. *

Service:
A sales representative will be pleased to assist you in the use of this product. For additional information technical support or service, please call (b) (6), (b) (4)

(b) (6), (b) (4)

Date: 07/08/20
Time: 14:56:17

(b) (4)

(b) (6), (b) (4)

(b) (4) Certificate of Analysis

Sold To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: (b) (4)
Customer Order No.:
Item No.: AMBEREX 1003 AG 40 LB BAG
40 LB BAG

Customer Item:
Lot No.: (b) (4) 1.000000 BG
Manufacture Date: 12/04/18
Lot Expiration Date: 12/03/20

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	30.0	100.0	32.5
Ash %	AOAC 930.30	0	16.0	14.8
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	1.0
pH (5% solution)	pH Meter	5.3	6.3	5.7
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	64.8
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE
Salt as Chlorides %	AOAC 971.27	0	1.50	0.6
Standard Plate Count cfu/g	AOAC 990.12	0	10000	100.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

Date: 06/18/19
Time: 14:32:38
Page 0 of 0

(b) (4)

(b) (6), (b) (4)

(b) (4) Certificate of Analysis

Sold To:

Customer PO No.:
Customer Order No.:
Item No.: (b) (4) AMBERFERM 7020 AG
18.14 KG/40 LB BAG
Customer Item:
Lot No.: (b) (4)
Manufacture Date: 03/12/19
Lot Expiration Date: 03/11/21

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	6.0	100.0	9.1
Ash %	AOAC 930.30	0	15.0	10.1
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.6
pH (5% solution)	pH Meter	5.2	6.2	5.8
Protein (N x 6.25) %	AOAC 990.03	70.0	100.0	74.7
Salt as Chlorides %	AOAC 971.27	0	2.00	0.27
Standard Plate Count cfu/g	AOAC 990.12	0	10000	10.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0
Salmonella /375g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

Date: 01/25/19
Time: 15:28:53

Page 0 of 0

(b) (4)

(b) (6), (b) (4)

(b) (4) Certificate of Analysis

Sold To: (b) (6), (b) (4)

Customer PO No.:
Customer Order No.:
Item No.:

Amberferm 4210
50 LB Carton w/ Liner

Customer Item:
Lot No.: (b) (4)
Manufacture Date: 01/15/19
Lot Expiration Date: 01/15/21

300.000000 CT

Test Identification	Method	Min Value	Max Value	Test Value
MOISTURE METTLER POWDER		0	6.0	3.8
PH (10% SOLUTION)		.5	5.5	4.8
SALT AS CHLORIDES %		0	2.5	1.1
AMINO NITROGEN/TOTAL NITROGEN		50.0	100.0	79.5
ASH		0	12.0	7.6
% EQUIV. PROTEIN (NX6.25)		74.0	100.0	78.6
FLAVOR		PASS	PASS	PASS
APPEAR		PASS	PASS	PASS
ODOR		PASS	PASS	PASS
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	<10000 /G
COLIFORM (CFU /G)		<10 /G	<10 /G	<10 /G
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	<100 /G
SALMONELLA ELFA METHOD 375G		ND	ND	ND
E. COLI MPN/g		ND	ND	ND

*ND = NOT DETECTED

(b) (6)

Date: 05/01/20
Time: 18:29:59
Page 0 of 0

(b) (4)

(b) (6), (b) (4)

(b) (4) Certificate of Analysis

Sold To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: (b) (4)
Customer Order No.:
Item No.: SENSIFERM GROW 605 40 LB BAG
40 LB BAG

Customer Item:
Lot No.: (b) (4) 1.000000 BG
Manufacture Date: 09/11/18
Lot Expiration Date: 09/10/21

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	5.0	100.0	7.0
Ash %	AOAC 930.30	0	20.0	16.9
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.7
pH (5% solution)	pH Meter	5.5	6.5	6.1
Salt as Chlorides %	AOAC 971.27	0	1.00	0.66
Standard Plate Count cfu/g	AOAC 990.12	0	10000	0.0
Yeast and Mold cfu/g	AOAC 121301	0	50	0.0
Salmonella /375g	AOAC OMA 2003.09	NEGATIVE	NEGATIVE	NEGATIVE
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	55.2

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

(b) (6)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Ferrous Sulfate, Heptahydrate, Granular, USP		
CAS Number	7782-63-0		
Molecular Formula	$FeSO_4.7H_2O$	Molecular Weight	278.02

Test	Specification		Result
	min	max	
ASSAY (as HEPTAHYDRATE)	99.5	104.5 %	100.0 %
ARSENIC		3 ppm	<3 ppm
LEAD		10 ppm	<1 ppm
MERCURY		3 µg/g	<1 µg/g
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
IDENTIFICATION	POSITIVE FOR IRON, FERROUS SALTS AND SULFATE		POSITIVE FOR IRON, FERROUS SALTS AND SULFATE
EXPIRATION DATE			01-JUN-2021
DATE OF MANUFACTURE			01-JUN-2018
APPEARANCE			PALE BLUE GREEN CRYSTALS
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Cysteine Hydrochloride, Monohydrate, USP		
CAS Number	7048-04-6		
Molecular Formula	$C_3H_7NO_2S.HCl.H_2O$	Molecular Weight	175.64

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.5	101.5 %	99.5 %
SPECIFIC ROTATION $[a]_D$	+5.7° to +6.8°		+5.95°
LOSS ON DRYING	8.0	12.0 %	10.12 %
RELATED COMPOUNDS:			.
INDIVIDUAL IMPURITY		0.5 %	<0.5 %
TOTAL IMPURITIES		2.0 %	<2.0 %
RESIDUE ON IGNITION		0.4 %	0.01 %
SULFATE		0.03 %	<0.03 %
IRON		30 ppm	<30 ppm
ELEMENTAL IMPURITIES	AS REPORTED		NO ELEMENTAL IMPURITIES PRESENT
IDENTIFICATION (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE CRYSTALS
EXPIRATION DATE			06-OCT-2021
DATE OF MANUFACTURE			07-OCT-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (6), (b) (4)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Magnesium Sulfate, Anhydrous, Powder, Reagent		
CAS Number	7487-88-9		
Molecular Formula	$MgSO_4$	Molecular Weight	120.37

Test	Specification		Result
	min	max	
ASSAY ($MgSO_4$)	99 %		99.12 %
AMMONIUM (NH_4)		0.005 %	<0.005 %
ARSENIC (As)		0.0001 %	0.0001 %
CALCIUM (Ca)		0.04 %	<0.04 %
CHLORIDE (Cl)		0.005 %	0.001 %
HEAVY METALS (as Pb)		0.001 %	0.001 %
IRON (Fe)		0.001 %	0.001 %
LOSS ON IGNITION		5.0 %	5.0 %
MANGANESE (Mn)		0.001 %	0.0005 %
NITRATE (NO_3)		0.005 %	<0.005 %
APPEARANCE			WHITE POWDER
DATE OF MANUFACTURE			16-AUG-2014

(b) (4), (b) (6)

(b) (6), (b) (4)

C5130 Maltose
Purified, FCC, Grade
Certificate of Analysis

Batch: tbd
Formula: $C_6H_{14}O_6$
Formula Wt: 360.31
CAS #: 6363-53-7
Country of Origin: USA

Grade: Purified, FCC
Manufacture Date: 8/1/2019
Batch Size: 2250kg
Expiration: 8/1/2024

Customer: tbd
Customer Part #: C5130
Order Date: na

Customer PO#: na
Ship Date: na

Analytical Results

TEST	SPECIFICATION	OBSERVATION
Assay: Maltose	≥92.0	>92.0%
Glucose	≤3.0%	<3.0%
Loss on Drying	≤7.0%	<7.0%
Heavy Metals, as Lead	≤5 ppm	<5.0 ppm

(b) (6)

(b) (6)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Manganese Sulfate, Monohydrate, Powder, FCC, BP		
CAS Number	10034-96-5		
Molecular Formula	$MnSO_4.H_2O$	Molecular Weight	169.02

Test	Specification		Result
	min	max	
ASSAY ($MnSO_4.H_2O$)	98.0	102.0%	99.95 %
ASSAY (IGNITED)	99.0	101.0%	100.49 %
LOSS ON HEATING	10.0	12.0%	11.15 %
APPEARANCE OF SOLUTION	TO PASS TEST		PASSES TEST
ARSENIC (As)		3 mg/kg	<3 mg/kg
LEAD (Pb)		4 mg/kg	0.6 mg/kg
SELENIUM (Se)		0.003%	<0.003 %
HEAVY METALS		20 ppm	<20 ppm
ELEMENTAL IMPURITIES		AS REPORTED	COMPLIES WITH STANDARD
IRON		10 ppm	<10 ppm
ZINC (Zn)		50 ppm	6 ppm
CHLORIDE (Cl)		100 ppm	<50 ppm
IDENTIFICATION		TO PASS TEST	PASSES TEST
RETEST DATE			15-APR-2021
DATE OF MANUFACTURE			16-APR-2016
APPEARANCE			PINK CRYSTALLINE POWDER
RESIDUAL SOLVENTS		TO PASS TEST	NO RESIDUAL SOLVENTS USED

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Potassium Phosphate Monobasic, FCC		
CAS Number	7778-77-0		
Molecular Formula	KH_2PO_4	Molecular Weight	136.09

Test	Specification		Result
	min	max	
ASSAY (KH_2PO_4; DRIED BASIS)	98.0 %		101.0 %
ARSENIC (As)		3 mg/kg	0.01 mg/kg
FLUORIDE		10 mg/kg	1.2 mg/kg
INSOLUBLE SUBSTANCES		0.2 %	0.00 %
LEAD (Pb)		2 mg/kg	0.01 mg/kg
LOSS ON DRYING		1 %	0.04 %
IDENTIFICATION	TO PASS TEST		PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			30-APR-2023
DATE OF MANUFACTURE			01-APR-2020
APPEARANCE			WHITE CRYSTALLINE POWDER
MONOGRAPH EDITION			(FCC) 11

(b) (6), (b) (4)

Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of *Ruminococcus bovis* ASCUSDY10 for Use in Animal Feed

Native Microbials

Aug 2023

Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

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Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

1. INTRODUCTION

Native Microbials, Inc. (hereafter referred to as "Native Microbials") develops direct-fed microbial (DFM) productsfor use as supplementary feeds for poultry and cattle in the United States (U.S.). One of the raw materials used to charge the fermenter for the production of the DFM strains is monopotassium phosphate, FCC grade. While dipotassium phosphate is permitted for use as a sequestrant in feed in accordance with good manufacturing or feeding practice under 21 CFR §582.6282[1], monopotassium phosphate is currently not currently acceptable for feeding to animals in the U.S. Considering that all raw materials used in the production of DFM products should be accepted feed substances in the U.S., Native Microbials has conducted a safety evaluation to confirm the suitability of monopotassium phosphate for the intended use as a processing aid in the fermentation of its microbial strains.

2. REGULATORY STATUS

2.1 Regulatory Status in Animal Feed in the U.S.

A number of related phosphate salts are acceptable for use in animal feed in the U.S. and are summarized in Table 2.1.

Table 2.1: Examples of Related Phosphate Salts Accepted for Use in Animal Feed in the U.S.

Mineral Substance	Function in Feed	Regulatory Status
Diammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.16
Dicalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.71
Disodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.32
Monoammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.33
Monocalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.98
Monosodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.99
Phosphoric acid	Mineral product and general purpose food additive	21 CFR §582.1073 and AAFCO ingredient definition 57.19
Dipotassium phosphate	Sequestrant	21 CFR §582.6282

[1] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=582.6285&SearchTerm=dipotassium%20phosphate

2.2 Regulatory Status in Animal Feed in Canada

Monopotassium phosphate is permitted for use in animal feed as in Canada as a Class 6 – Mineral Product under Schedule IV, Part I of the Feed Regulations (1983). The substance must be labelled with guarantees for minimum percent potassium, minimum percent phosphorus and maximum milligrams fluorine, arsenic and iron per kilogram

2.3 Regulatory Status in Animal Feed in the European Union (EU)

Monopotassium phosphate is a recognized feed material in the EU and listed in the Feed Materials Catalogue laid down under Commission Regulation (EU) No 68/2013 (European Commission, 2013). The substance must be labelled with total phosphorus, potassium and, where greater than 10%, the content of phosphorus insoluble in citric acid.

2.4 Regulatory Status in Human Food in the U.S.

Monopotassium phosphate is generally recognized as safe as a food additive in frozen eggs at levels of less than 0.5% in accordance with 21 CFR §160.110.

3. SAFETY EVALUATION FOR TARGET ANIMALS

3.1 History of Use

As mentioned in Section 2, monopotassium phosphate has a long and established history of use as a mineral substance for use in animal feed in Canada and the EU. The levels of monopotassium phosphate as a source of phosphorus in feed is expected to be higher than the residues arising from carry-over of the fermentation process in DFM products. On this basis, the history of safe use of monopotassium phosphate in Canada and the EU for use in animal feed supports the suitability of the additive for use as a raw material in the fermentation of microbial strains by Native Microbials.

3.2 Natural Occurrence

Potassium is present in most feedstuffs with the highest levels typically reported in protein sources such as soybean meal. Thus, deficiencies in animals, particularly non ruminants are rare (NRC, 2005). Where diets contain high levels of industrial by-products such as brewer's grains or corn gluten, supplementation can be required.

Likewise, phosphates are widely available from the feed, with oilseed meals and other plant-based materials, mineral feeds, and meat and marine animal feeds serving as major sources in the diet of animals. Availability of phosphorus from the diet can vary with the source and is generally taken into account in the formulation of livestock diets (NRC, 2005).

It is reasonable to assume that these background sources will provide potassium and phosphorus as significantly higher levels in the diet of poultry and cattle than will be carried over from the use as a fermentation aid in the production of microbial strains by Native Microbials.

3.3 Metabolic Fate

On ingestion by animals, monopotassium phosphate will dissociate to the respective potassium, hydrogen and phosphate ions. Equivalent behaviour in the gastrointestinal tract is observed on ingestion

of related salts such as mono- and di-sodium phosphate and dipotassium phosphate. Thus, the use of monopotassium phosphate will result in exposure by animals to ions commonly consumed in animal feed. On this basis, the available safety data on sodium, calcium and ammonium phosphate salts as well as dipotassium phosphate may be extrapolated to support the safety of monopotassium phosphate (see Section 3.3 and 3.4).

3.4 Mineral Tolerances

Both potassium and phosphorus are required nutrients for poultry and cattle and are considered by the National Research Council (NRC) to be of medium concern for animal health. The NRC has set maximum tolerable levels for potassium of 1% in the diet of poultry and cattle on a dry matter basis, and for phosphorus of 1% for growing birds, 0.8% for laying hens and 0.7% for cattle on a dry matter basis (NRC, 2005). Any carry-over in the diet of monopotassium phosphate from the production of microbial strains for use as DFM products will contribute to the levels of these minerals in the feed but the overall impact on the daily intakes by animals is expected to be very low.

3.5 Evaluations by Scientific Bodies

3.5.1 JECFA Evaluation

The Joint FAO/WHO Committee on Food Additives (JECFA) has evaluated the safety of phosphoric acid and phosphate salts as a group, including within the scope of the review, mono-, di- and tri-potassium phosphate (JECFA, 1982). In the latest evaluation conducted in 1982, JECFA concluded that:

"*Metabolically, the phosphate salts provide a source of the various cations and phosphate ion. Of the greatest concern is the toxicity arising from calcium, magnesium and phosphate imbalance in the diet. Phosphate salts were not mutagenic in a number of test systems. Teratogenic effects have not been observed in mammalian test systems.*

Numerous animal studies have shown that excessive dietary phosphorus causes an increase of plasma phosphorus and a decrease in serum calcium. The resulting hypocalcaemia stimulates excretion of PTH which in turn increases the rate of bone resorption and decreases calcium excretion. These homeostatic adjustment to high dietary phosphorus may result in bone loss and calcification of soft tissues in animals.

The dose levels of phosphate producing nephrocalcinosis were not consistent among the various rat feeding studies. However, the rat is exquisitely susceptible to calcification and hydronephrosis upon exposure to acids forming calcium chelates or complexes. The lowest dose levels that produce nephrocalcinosis overlap the higher dose levels failing to do so. However, this may be related to other dietary imbalances, such as the level of magnesium in the diet. There is still uncertainty on the optimal Ca:P ratio and whether this ratio is of any dietary significance in man.

The lowest level of phosphate that produced nephrocalcinosis in the rat (1% P in the diet) is used as the basis for the evaluation and, by extrapolation based on the daily food intake of 2800 calories, this gives a dose level of 6600 mg P per day as the best estimate of the lowest level that might conceivably cause nephrocalcinosis in man. The usual calculation for provision of a margin of safety is probably not suitable for food additives which are also nutrients. Ingested phosphates from natural sources should be considered together with that from food additive sources. Since phosphorus (as phosphates) is an

essential nutrient and an unavoidable constituent of food, it is not feasible or appropriate to give a range of values from zero to maximum."

On the basis of the above, the maximum tolerable daily intake for man was estimated to be 70 mg/kg body weight.

3.5.2 SCF Evaluation

The Scientific Committee on Food (SCF) in the European Union (EU) evaluated the group of phosphate salts used as food additives in 1990 and agreed with the JECFA estimate of 70 mg/kg body weight for man, calculated as phosphorus (SCF, 1990).

3.5.3 Summary

Taken together the body of available data indicate that the safety of monopotassium phosphate can be considered from the available data on phosphoric acid and phosphate, which have been previously evaluated by JECFA and the SCF for use as food additives. These evaluations highlighted the role of phosphate salts to provide a metabolic source of cations and the phosphate ion. Safety was primarily based on the absence of any genotoxicity and the requirement to provide nutritionally balanced levels in the diet which do not exceed the maximum that can be tolerated by the body.

4. EXPOSURE ANALYSIS

(b) (4)

5. SUMMARY AND CONCLUSIONS

Monopotassium phosphate has an established history of safe use as a mineral substance for use in animal feed in Canada and in the EU. On ingestion by poultry or cattle, monopotassium phosphate will dissociate into the potassium, hydrogen and phosphate ions. For this reason, and consistent with the evaluations of the additive for use in food by JECFA and the SCF, the safety can be primarily derived from the body of available data on phosphoric acid and phosphate salts. Potassium and phosphate are both essential nutrients for animals and present naturally in the feed as well as being added in the form of supplemental salts. The carry-over of potassium and phosphate from its use as a monopotassium salt in the fermentation of microbial strains for use as DFMs in poultry and cattle feed is shown in the example above to make insignificant contribution to the levels present in the diet from natural and supplemental sources.

Together, it is concluded that there are no safety concerns associated with the use of monopotassium phosphate by Native Microbials as a fermentation aid under the conditions of intended use.

6. REFERENCES

CIR, 2016. Cosmetic Ingredient Review. Phosphoric acid and simple salts as used in cosmetics. Available at: https://www.cir-safety.org/

JECFA, 1982. Joint FAO/WHO Expert Committee on Food Additives. Toxicological Monograph: Phosphoric acid and phosphate salts. Available at: http://www.inchem.org/documents/jecfa/jecmono/v17je22.htm

NRC, 1990. National Research Council. Mineral Tolerances of Animals. The National Academies Press.

SCF, 1990. Scientific Committee on Food. Report, 25th Series. Food additives of various technological functions. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_reports_25.pdf

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (6)
Item	Niacin, Powder, USP		
CAS Number	59-67-6		
Molecular Formula	$C_6H_5NO_2$	Molecular Weight	123.11

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.0%	102.0%	100.0 %
LOSS ON DRYING		1.0 %	0.1 %
RESIDUE ON IGNITION		0.1 %	<0.1 %
CHLORIDE (Cl)		0.02 %	<0.02 %
SULFATES (SO_4)		0.02 %	<0.02 %
ELEMENTAL IMPURITIES:			.
CADMIUM (Cd)		AS REPORTED	<0.001 µg/g
LEAD (Pb)		AS REPORTED	<0.001 µg/g
ARSENIC (As)		AS REPORTED	<0.01 µg/g
MERCURY (Hg)		AS REPORTED	<0.01 µg/g
RELATED COMPOUNDS		TO PASS TEST	PASSES TEST
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE PWDER
EXPIRATION DATE			20-JUN-2022
DATE OF MANUFACTURE			21-JUN-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (6), (b) (4)

(b) (4)

(b) (4)

(b) (4)

Product Type	**FOOD GRADE – GENERAL PURPOSE PROCESS AID DEFOAMER**
Product Description	(b) (4) is a defoamer designated to control foam in many processes. is especially effective when used in fermentation processes where a certain degree of foam control is needed without affecting oxygen transfer for optimum product yield. This product is made with food grade ingredients under our Good Manufacturing Practices Program. The components of (b) (4) meet FDA requirement for use in egg washing, potato processing defoamers as a dispersing aid for mineral oil at a limit of 10 ppm in the processing water followed by a potable water rinse. This product also contains ingredients for which the FDA has provided the Enzyme Technical Association with a "no objection" letter acknowledging that they are used as defoaming agents in the manufacture of enzyme preparations used in food in accordance with the principles of GMPs. Other uses in the processing and manufacture of food ingredients may also qualify for GRAS status. (b) (4) also is composed of ingredients that meet the current requirements of the FDA for food contact applications when used in accordance with the requirements and limitations of 21CFR 176.210(d)(3). Consideration for other FDA permitted uses would require further evaluation.

Typical Properties

Appearance	Clear Liquid
Viscosity @ 100°F, Kinematic	185 – 210 Cst
Odor	Sweet
Weight per gallon	8.5 Lbs
Flash Point (°C)	> 216°C PMCC (Min)
Specific Gravity	1.02

Typical Applications

Typical applications for (b) (4) include:

- Fermentation
- Egg washing

Incorporation

(b) (4) should be added, as received, early in the processing to prevent foam before it forms. (b) (4) should be evaluated in the process to determine the optimum dosage and legal limits allowed.

Shelf Life

2 years from date of manufacture when properly stored in the original container following proper storage and handling.

Storage & Handling

Keep from freezing. Store product between 40 and 100°F. Keep containers tightly closed when not in use.

Responsible Care

For complete safety, health, personnel protection and first aid information, refer to the Safety Data Sheet (SDS) that can be ordered through the numbers below.

18, 2017

(b) (6), (b) (4)

(b) (4)

April 14, 2021

Native Microbials

SUBJECT: FDA 21 CFR COMPLIANCE – (b) (4)

To whom it may concern,

This product complies with the United State Food and Drug Administration's Code of Federal Regulations Title 21-Part 173.340, Secondary Direct Food Additives Permitted in Food for Human Consumption when used as a defoaming agent and its ingredients are listed under §173.340(a)(2).

The composition of (b) (4) Control's product (b) (4) is described as (b) (4).

We hope this information is useful to you. If you should have any further questions, please feel free to contact us.

Sincerely,

(b) (6)

Operation Manager

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Pyridoxine Hydrochloride, USP		
CAS Number	58-56-0		
Molecular Formula	$C_8H_{11}NO_3$.HCl	Molecular Weight	205.64

Test	Specification		Result
	min	max	
ASSAY	98.0	102.0 %	100.0 %
CHLORIDE CONTENT	16.9	17.6 %	17.2 %
LOSS ON DRYING		0.5 %	0.00 %
RESIDUE ON IGNITION		0.1 %	0.02 %
ELEMENTAL IMPURITIES:	:		.
LEAD (Pb)	AS REPORTED		<0.5 µg/g
CADMIUM (Cd)	AS REPORTED		<0.5 µg/g
ARSENIC (As)	AS REPORTED		<0.5 µg/g
MERCURY (Hg)	AS REPORTED		<0.1 µg/g
IDENTIFICATION A		TO PASS TEST	PASSES TEST
IDENTIFICATION B		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
RETEST DATE			17-MAR-2024
DATE OF MANUFACTURE			18-MAR-2020
APPEARANCE			WHITE CRYSTALLINE POWDER
RESIDUAL SOLVENTS		TO PASS TEST	PASSES TEST
CLASS 3 (solvent) / ETHANOL			<5000 ppm
MONOGRAPH EDITION			(USP) 42

(b) (6), (b) (4)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Riboflavin, USP		
CAS Number	83-88-5		
Molecular Formula	$C_{17}H_{20}N_4O_6$	Molecular Weight	376.36

Test	Specification		Result
	min	max	
ASSAY ($C_{17}H_{20}N_4O_6$)	98.0%	102.0 %	98.4 %
SPECIFIC ROTATION $[a]_D$	-115° to -135°		-120.0°
LOSS ON DRYING		1.5 %	0.3 %
RESIDUE ON IGNITION		0.3 %	0.10 %
LUMIFLAVIN		0.025	0.004
ELEMENTAL IMPURITIES:			.
CADMIUM (Cd)	AS REPORTED		<1 µg/g
LEAD (Pb)	AS REPORTED		<0.5 µg/g
ARSENIC (As)	AS REPORTED		<1 µg/g
MERCURY (Hg)	AS REPORTED		<0.1 µg/g
IDENTIFICATION	PALE GREENISH YELLOW WITH YELLOWISH-GREEN FLUORESCENCE		PALE GREENISH YELLOW WITH YELLOWISH-GREEN FLUORESCENCE
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			ORANGE POWDER
EXPIRATION DATE			01-MAR-2022
DATE OF MANUFACTURE			02-MAR-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (6), (b) (4)

(b) (4)

Date: 07/08/20
Time: 14:56:17

(b) (4)

(b) (6), (b) (4)

(b) (4) Certificate of Analysis

Sold To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: (b) (4)
Customer Order No.:
Item No.: AMBEREX 1003 AG 40 LB BAG
40 LB BAG

Customer Item:
Lot No.: (b) (4) 1.000000 BG
Manufacture Date: 12/04/18
Lot Expiration Date: 12/03/20

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	30.0	100.0	32.5
Ash %	AOAC 930.30	0	16.0	14.8
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	1.0
pH (5% solution)	pH Meter	5.3	6.3	5.7
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	64.8
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE
Salt as Chlorides %	AOAC 971.27	0	1.50	0.6
Standard Plate Count cfu/g	AOAC 990.12	0	10000	100.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

Date: 06/18/19
Time: 14:32:38
Page 0 of 0

(b) (4)

(b) (4) Certificate of Analysis

(b) (6), (b) (4)

Sold To:

Customer PO No.:
Customer Order No.:
Item No.: (b) (4) AMBERFERM 7020 AG
18.14 KG/40 LB BAG
Customer Item:
Lot No.: (b) (4)
Manufacture Date: 03/12/19
Lot Expiration Date: 03/11/21

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	6.0	100.0	9.1
Ash %	AOAC 930.30	0	15.0	10.1
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.6
pH (5% solution)	pH Meter	5.2	6.2	5.8
Protein (N x 6.25) %	AOAC 990.03	70.0	100.0	74.7
Salt as Chlorides %	AOAC 971.27	0	2.00	0.27
Standard Plate Count cfu/g	AOAC 990.12	0	10000	10.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0
Salmonella /375g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

Date: 01/25/19
Time: 15:28:53

Page 0 of 0

(b) (4)

(b) (4) Certificate of Analysis

(b) (6), (b) (4)

Sold To: (b) (6), (b) (4)

Customer PO No.:
Customer Order No.: (b) (4)
Item No.:

Amberferm 4210
50 LB Carton w/ Liner

Customer Item:
Lot No.: (b) (4)
Manufacture Date: 01/15/19
Lot Expiration Date: 01/15/21

300.000000 CT

Test Identification	Method	Min Value	Max Value	Test Value
MOISTURE METTLER POWDER		0	6.0	3.8
PH (10% SOLUTION)		.5	5.5	4.8
SALT AS CHLORIDES %		0	2.5	1.1
AMINO NITROGEN/TOTAL NITROGEN		50.0	100.0	79.5
ASH		0	12.0	7.6
% EQUIV. PROTEIN (NX6.25)		74.0	100.0	78.6
FLAVOR		PASS	PASS	PASS
APPEAR		PASS	PASS	PASS
ODOR		PASS	PASS	PASS
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	<10000 /G
COLIFORM (CFU /G)		<10 /G	<10 /G	<10 /G
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	<100 /G
SALMONELLA ELFA METHOD 375G		ND	ND	ND
E. COLI MPN/g		ND	ND	ND

(b) (6)

*ND = NOT DETECTED

Date: 05/01/20
Time: 18:29:59
Page 0 of 0

(b) (4)

(b) (4) Certificate of Analysis

(b) (6), (b) (4)

d To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: (b) (4)
Customer Order No.:
Item No.:

SENSIFERM GROW 605 40 LB BAG
40 LB BAG

Customer Item:
Lot No.: (b) (4)
Manufacture Date: 09/11/18
Lot Expiration Date: 09/10/21

1.000000 BG

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	5.0	100.0	7.0
Ash %	AOAC 930.30	0	20.0	16.9
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
steria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.7
pH (5% solution)	pH Meter	5.5	6.5	6.1
Salt as Chlorides %	AOAC 971.27	0	1.00	0.66
Standard Plate Count cfu/g	AOAC 990.12	0	10000	0.0
Yeast and Mold cfu/g	AOAC 121301	0	50	0.0
Salmonella /375g	AOAC OMA 2003.09	NEGATIVE	NEGATIVE	NEGATIVE
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	55.2

(b) (6), (b) (4)

(b) (6)

*ND = NOT DETECTED

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sodium Acetate, Anhydrous, USP	Manufacturer Lot	(b) (4)
CAS Number	127-09-3	Manufacturer Code	14941
Molecular Formula	$C_2H_3NaO_2$	Molecular Weight	82.03

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	99.0	101.0 %	100.1 %
pH OF A 3% SOLUTION @ 25°C	7.5	9.2	8.5
LOSS ON DRYING		1.0 %	0.03 %
INSOLUBLE MATTER		0.05 %	0.001 %
CHLORIDE (Cl)		350 ppm	< 350 ppm
SULFATES (SO_4)		50 ppm	< 50 ppm
CALCIUM AND MAGNESIUM	NO TURBIDITY		NO TURBIDITY
POTASSIUM (K)	NO PRECIPITATE		NO PRECIPITATE
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
IDENTIFICATION (A)	POSITIVE FOR SODIUM		POSITIVE FOR SODIUM
IDENTIFICATION (B)	POSITIVE FOR ACETATE		POSITIVE FOR ACETATE
EXPIRATION DATE			30-NOV-2021
DATE OF MANUFACTURE			01-MAY-2020
APPEARANCE			WHITE GRANULAR
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (6), (b) (4)

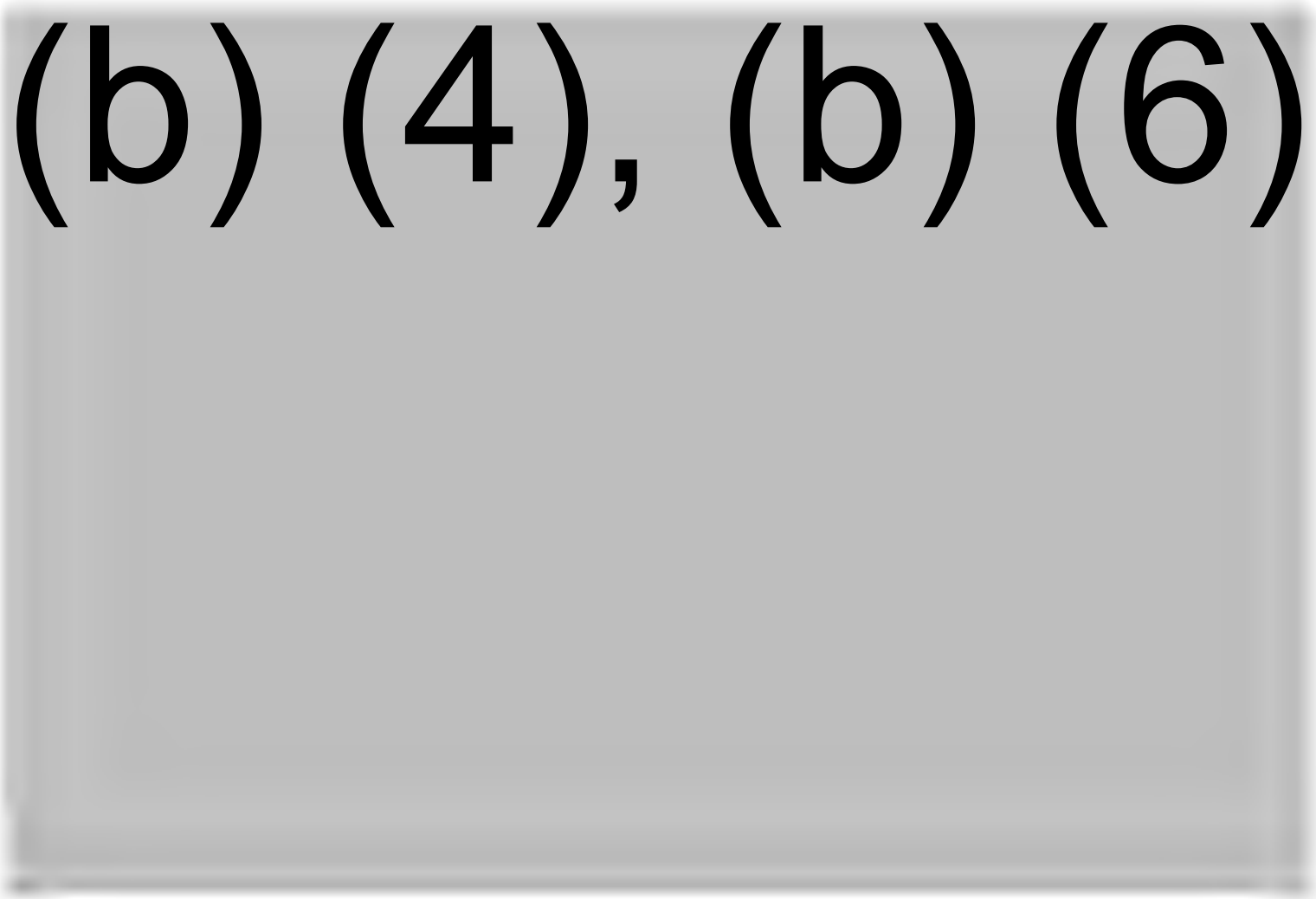
(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sodium Chloride, Granular, USP	Manufacturer Lot	(b) (4)
CAS Number	7647-14-5	Manufacturer Code	12349
Molecular Formula	NaCl	Molecular Weight	58.44

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	99.0	100.5 %	99.5 %
APPEARANCE OF SOLUTION	CLEAR COLORLESS		CLEAR COLORLESS
ACIDITY OR ALKALINITY		0.5 ml	<0.5 ml
LOSS ON DRYING		0.5%	0.1 %
ALUMINUM		0.2 ppm	<0.05 ppm
BROMIDES		100 ppm	<100 ppm
PHOSPHATES		25 ppm	<25 ppm
POTASSIUM		500 ppm	32 ppm
IODIDES	NO BLUE COLOR		NO BLUE COLOR
MAGNESIUM AND ALKALINE-EARTH METALS (as Ca)		100 ppm	4 ppm
ARSENIC (As)		1 ppm	<1 ppm
IRON (Fe)		2 ppm	<1 ppm
BARIUM (Ba)	OPALESCENCE LESS THAN REFERENCE		OPALESCENCE LESS THAN REFERENCE
FERROCYANIDES	NO BLUE COLOR		NO BLUE COLOR
SULFATE (SO_4)		200 ppm	<200 ppm
NITRITES		0.01	0.00
BACTERIAL ENDOTOXINS		5 IU/g	<2.5 IU/g
ELEMENTAL IMPURITIES	AS REPORTED		NO ELEMENTAL IMPURITIES PRESENT
IDENTIFICATION (A)	POSITIVE FOR SODIUM		POSITIVE FOR SODIUM
IDENTIFICATION (B)	PRECIPITATE DISSOLVES		PRECIPITATE DISSOLVES
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE GRANULES
RETEST DATE			09-JUL-2023
DATE OF MANUFACTURE			09-JUL-2020
RESIDUAL SOLVENTS	-AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42


(b) (4), (b) (6)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sodium Hydroxide, Pellets, FCC		
CAS Number	1310-73-2		
Molecular Formula	NaOH	Molecular Weight	40.00

Test	Specification		Result
	min	max	
ASSAY (TOTAL ALKALI as NaOH)	95.0 - 100.5 %		98.05 %
ARSENIC (As)		3 mg/kg	<3 mg/kg
CARBONATE (as Na_2CO_3)		3.0 %	0.55 %
INSOLUBLE SUBSTANCES & ORGANIC MATTER		TO PASS TEST	PASSES TEST
LEAD (Pb)		2 mg/kg	<2 mg/kg
MERCURY		0.1 mg/kg	<0.1 mg/kg
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED HALAL			HALAL
EXPIRATION DATE			26-APR-2021
DATE OF MANUFACTURE			27-APR-2016
APPEARANCE			WHITE PELLET

(b) (4), (b) (6)

Specifications for Sodium Sulfate

Ingredient:	Sodium Sulfate
Chemical Nomenclature:	$NaSO_4$
Specifications:	Feed Grade
Moisture:	≤ 1% by LOD
Purity:	≥ 98%

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sorbitol, Powder, FCC		
CAS Number	50-70-4		
Molecular Formula	$C_6H_{14}O_6$	Molecular Weight	182.17

Test	Specification		Result
	min	max	
ASSAY (ANHYDROUS BASIS)	91.0	100.5%	97.7 %
pH OF A 10% (w/w) SOLUTION	3.5	7.0	5.2
LEAD (Pb)		1 mg/kg	<1 mg/kg
NICKEL (Ni)		1 mg/kg	<1 mg/kg
REDUCING SUGARS		0.3%	<0.3 %
RESIDUE ON IGNITION		0.1 %	<0.1 %
WATER		1.5%	0.34 %
IDENTIFICATION	TO PASS TEST		PASSES TEST
RETEST DATE			12-OCT-2021
DATE OF MANUFACTURE			01-OCT-2019
APPEARANCE			WHITE POWDER

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sucrose, Crystal, NF		
CAS Number	57-50-1		
Molecular Formula	$C_{12}H_{22}O_{11}$	Molecular Weight	342.30

Test	Specification		Result
	min	max	
APPEARANCE OF SOLUTION	NO MORE OPALESCENCE THAN STANDARD		NO MORE OPALESCENCE THAN STANDARD
SPECIFIC ROTATION $[a]_D20$	+66.3 to +67.0°		+66.6°
CONDUCTIVITY @ 20 C		35 µS/cm	10 µS/cm
COLOR VALUE		75	52
LOSS ON DRYING		0.1 %	0.03 %
SULFITE		10 PPM	< 10 PPM
REDUCING SUGARS	BLUE COLOR DOES NOT DISAPPEAR COMPLETELY		BLUE COLOR DOES NOT DISAPPEAR COMPLETELY
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES TO STANDARD
IDENTIFICATION (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
CERTIFIED HALAL			CERTIFIED HALAL
RETEST DATE			28-FEB-2022
DATE OF MANUFACTURE			29-FEB-2020
APPEARANCE			WHITE CRYSTALS
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(NF) 37

(b) (4), (b) (6)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Vitamin B12, FCC		
CAS Number	68-19-9		
Molecular Formula	$C_{63}H_{88}CoN_{14}O_{14}P$	Molecular Weight	1355.37

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	96.0	100.5 %	98.6 %
LOSS ON DRYING		12.0 %	2.4 %
PSEUDO CYANOCOBALAMIN		TO PASS TEST	PASSES TEST
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			09-MAY-2022
DATE OF MANUFACTURE			10-MAY-2017
APPEARANCE			DARK RED POWDER

(b) (6), (b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Thiamine Hydrochloride, FCC		
CAS Number	67-03-8		
Molecular Formula	$C_{12}H_{17}ClN_4OS.HCl$	Molecular Weight	337.27

Test	Specification		Result
	min	max	
ASSAY ($C_{12}H_{17}ClN_4OS.HCl$; ANHYDROUS BASIS)	98.0	102.0 %	99.6 %
COLOR OF SOLUTION		TO PASS TEST	PASSES TEST
pH OF A 1 IN 100 SOLUTION	2.7 - 3.4		3.0
LEAD (Pb)		2 mg/kg	<2 mg/kg
NITRATE (NO_3)		TO PASS TEST	PASSES TEST
RESIDUE ON IGNITION		0.2 %	0.05 %
WATER		5.0 %	2.1 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			23-AUG-2020
DATE OF MANUFACTURE			24-AUG-2017
APPEARANCE			WHITE POWDER

(b) (4), (b) (6)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Zinc Sulfate, Heptahydrate, Granular, USP		
CAS Number	7446-20-0		
Molecular Formula	$ZnSO_4.7H_2O$	Molecular Weight	287.56

Test	Specification		Result
	min	max	
ASSAY (as Zinc Sulfate heptahydrate)	99.0	108.7 %	101.2 %
ACIDITY	NO PINK COLOR		NO PINK COLOR
ALKALIES AND ALKALI EARTH		0.9 %	0.3 %
ARSENIC (As)		14 ppm	<14 ppm
LEAD (Pb)		20 ppm	<20 ppm
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
IDENTIFICATION (A)	POSITIVE FOR ZINC		POSITIVE FOR ZINC
IDENTIFICATION (B)	POSITIVE FOR SULFATE		POSITIVE FOR SULFATE
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
RETEST DATE			03-APR-2022
DATE OF MANUFACTURE			03-APR-2019
APPEARANCE			WHITE CRYSTALS
RESIDUAL SOLVENTS		AS REPORTED	NO RESIDUAL SOLVENTS USED

(b) (4), (b) (6)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	(b) (4)
Item	Sulfuric Acid, FCC		
CAS Number	7664-93-9		
Molecular Formula	H_2SO_4	Molecular Weight	98.08

Test	Specification		Result
	min	max	
ASSAY	95.0 - 98.0 %		96.1 %
ARSENIC (As)		3 mg/kg	<1 mg/kg
CHLORIDE (Cl)		0.005 %	<0.005 %
IRON (Fe)		0.02 %	<0.01 %
LEAD (Pb)		5 mg/kg	<5 mg/kg
NITRATE (NO_3)		10 mg/kg	<10 mg/kg
REDUCING SUBSTANCES (as SO)		TO PASS TEST	PASSES TEST
SELENIUM (Se)		0.002 %	<0.002 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
APPEARANCE			CLEAR COLORLESS LIQUID
DATE OF MANUFACTURE			28-SEP-2015

(b) (4), (b) (6)

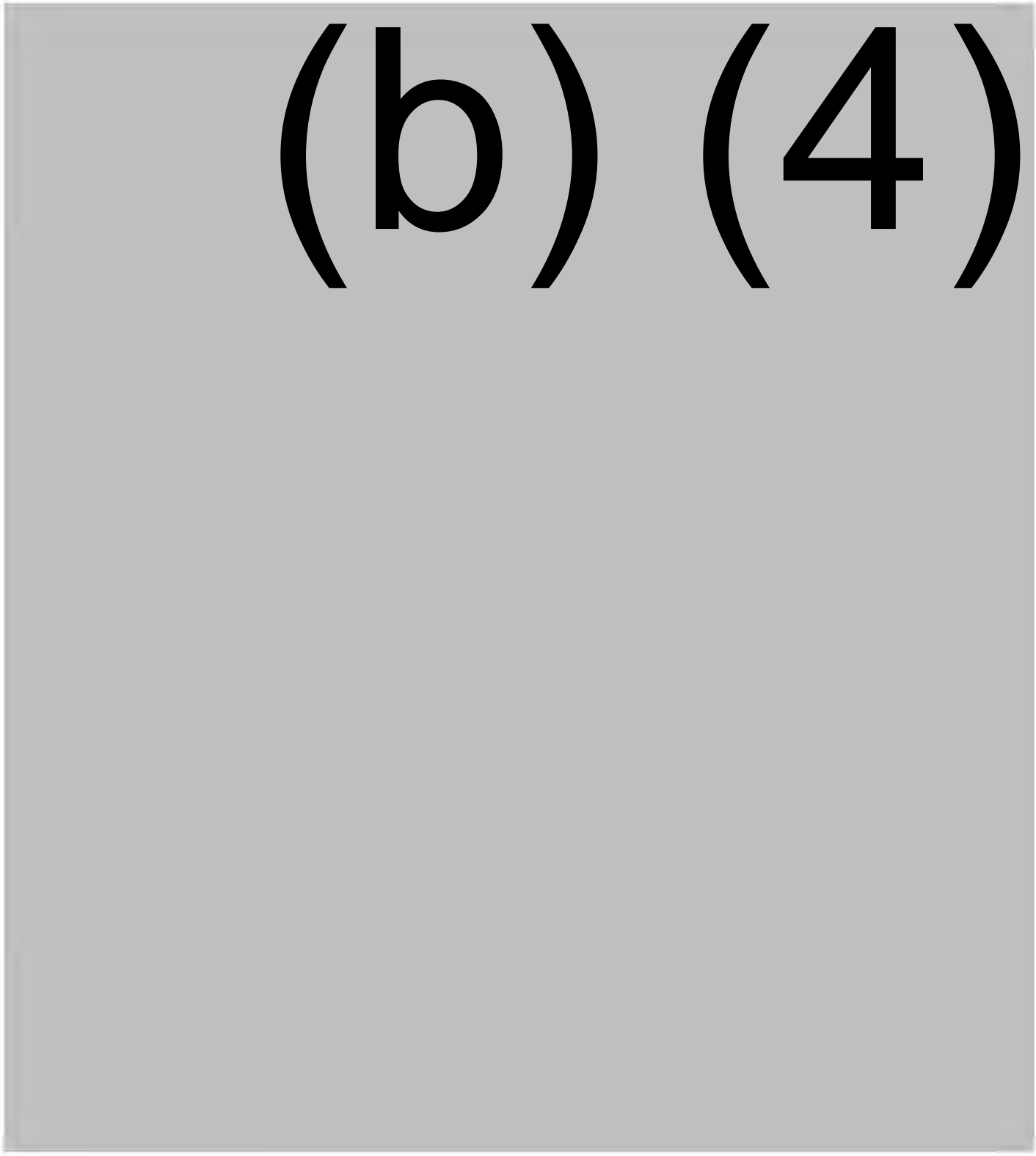
Confidential Detailed Manufacturing Summary of Fat Encapsulated *Ruminococcus bovis* ASCUSDY10

Confidential Manufacturing Information

The raw materials used in the manufacture of *R. bovis* ASCUSDY10 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009ZF.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *R. bovis* ASCUSDY10

(b) (4)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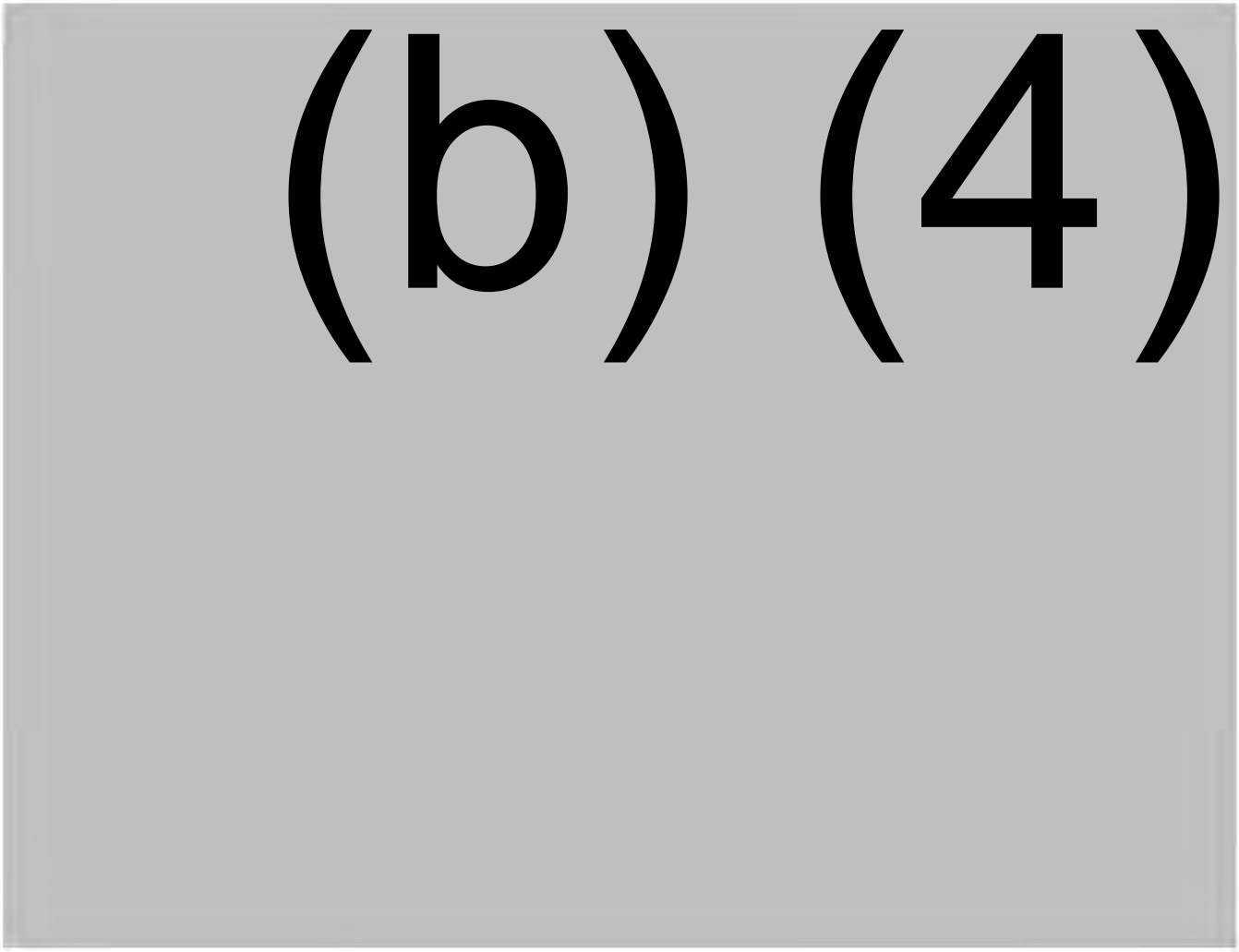

(b) (4)

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Ruminococcus bovis* ASCUSDY10

1 Overview

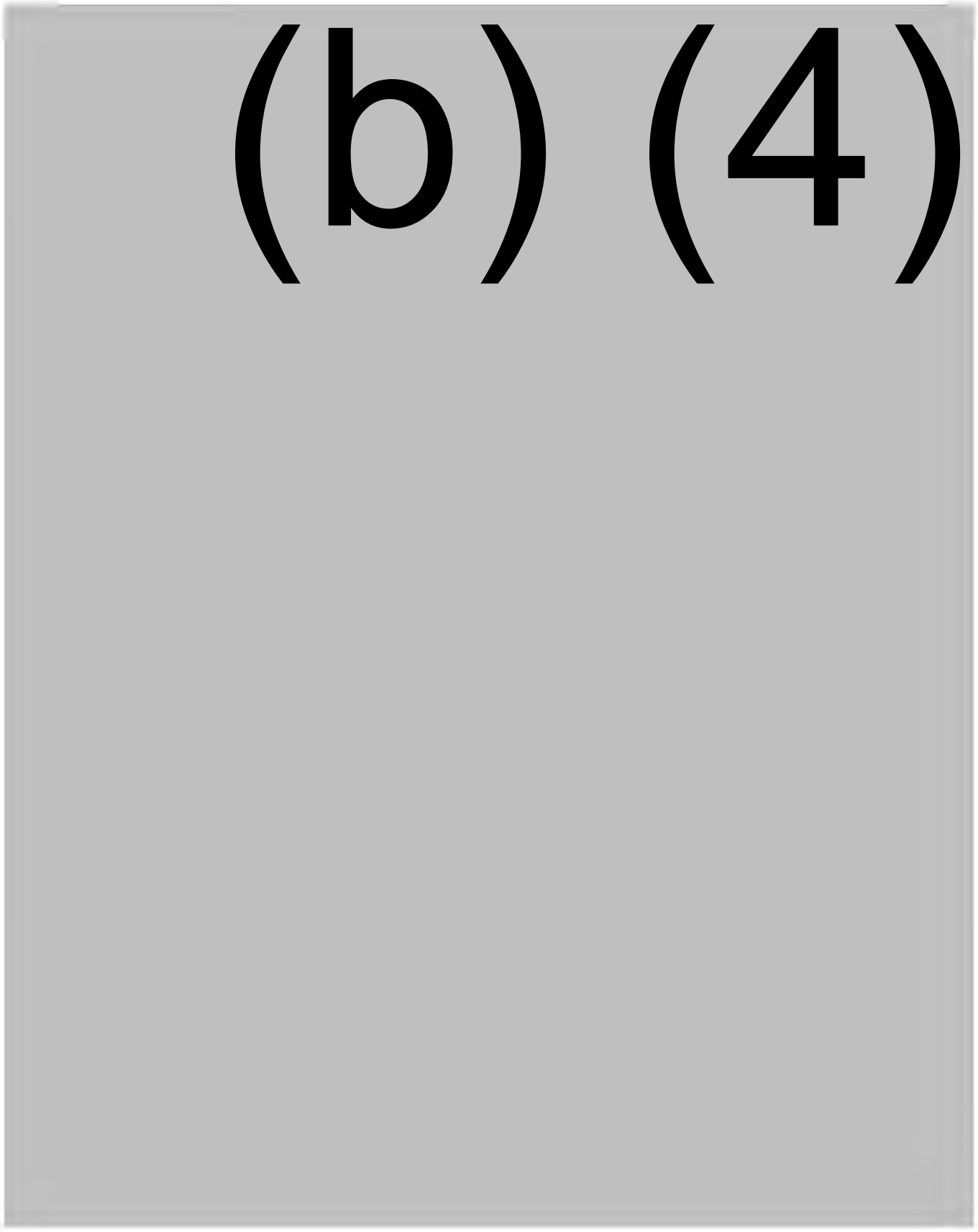
(b) (4)

2 Master Cell Bank / Working Cell Bank

(b) (4)

3 Fermentation

(b) (4)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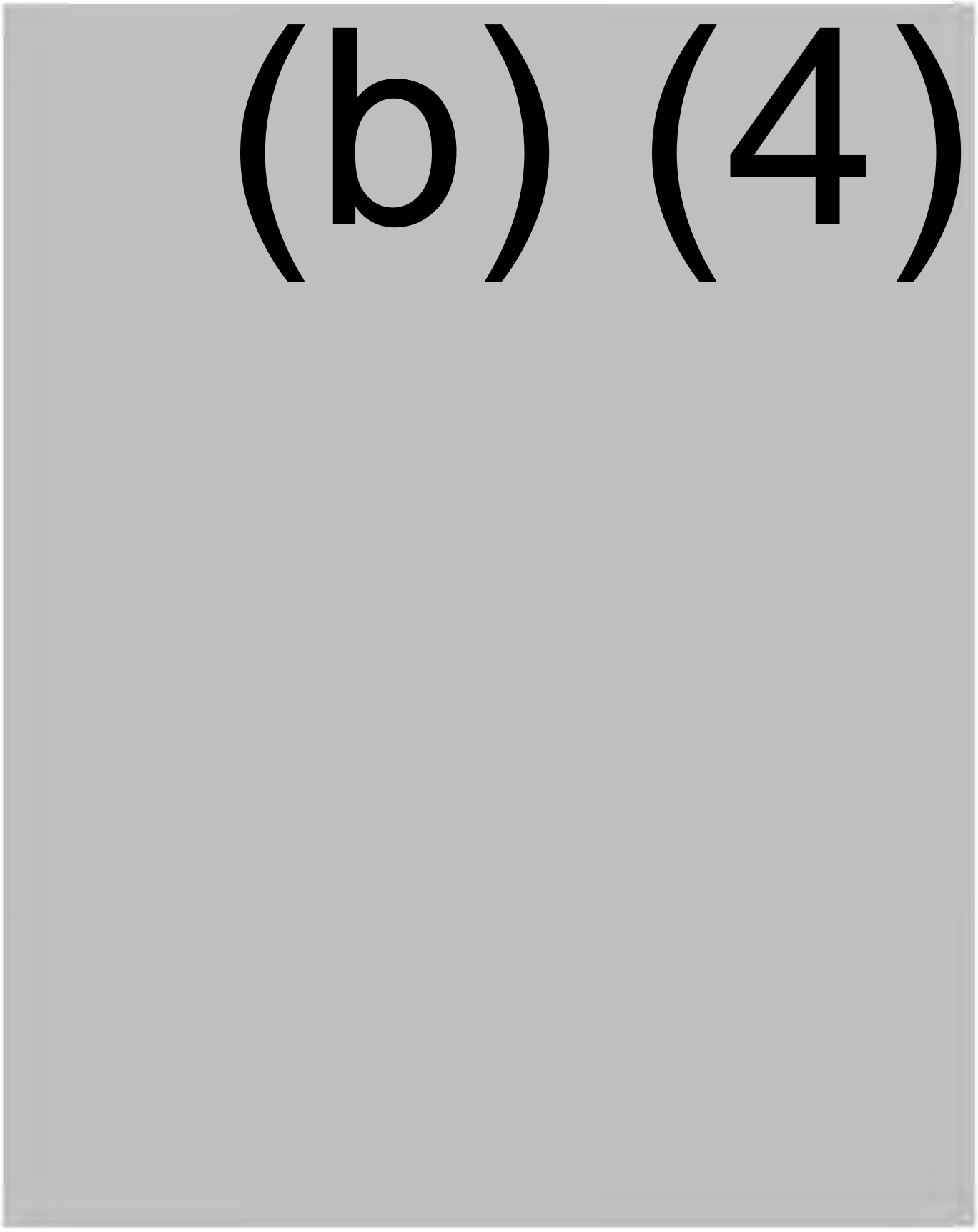
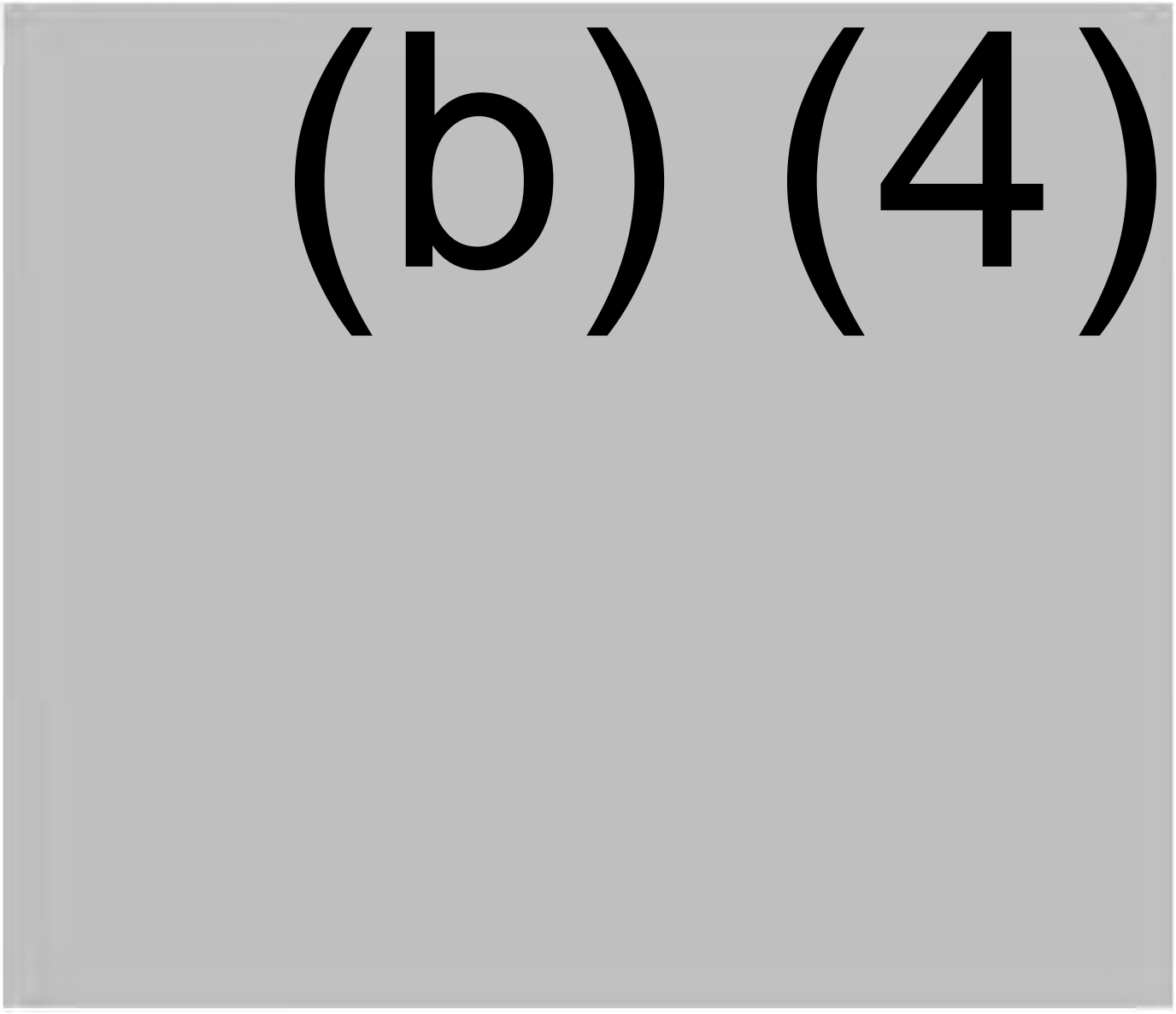
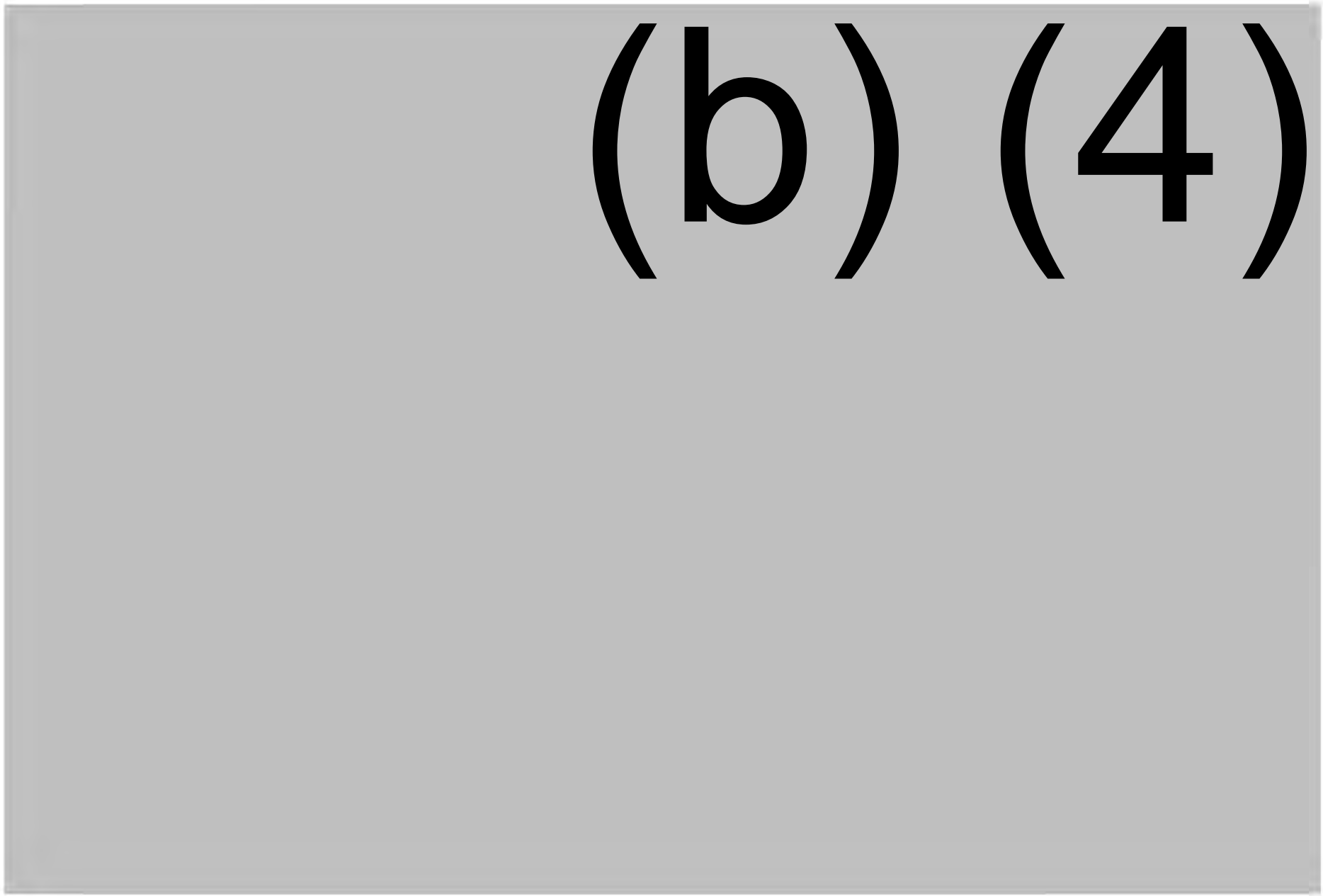

(b) (4)

(b) (4)

(b) (4)


(b) (4)


(b) (4)

Appendix 011

Comparison of Physical Properties of Fat Encapsulated Powder *R. bovis* ASCUSDY10 to recent prior submissions (AGRN 42) *Buyrivibrio fibrisolvens* ASCUSDY19

Physical Attribute	*R. bovis* ASCUSDY10	*B. fibrisolvens* ASCUSDY19	Method
Organism concentration	(b) (4)		Internal Methods (Appendices 012C & ARGN 42 Appendix 012C)
Particle size (d_{50})			Laser Diffraction
Particle size (d_{90})			Laser Diffraction
Milled foam dried organism composition (g/kg in in final formula)			By addition
Sodium Sulfate composition (g/kg in final formula)			By addition
Hydrogenated glycerides composition (g/kg in final formula)			By addition
Moisture content			Internal Method (Appendix 012D)

Method Validation Protocol, Version 1

Method Title and Versions

Title	DY10 Solid Intermediate Microbe Enumeration
Version	01

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b) (6)	11/13/2020
(b) (6) – Quality	(b) (6)	11/13/2020

Post Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b) (6)	12/1/2020
(b) (6) - Quality	(b) (6)	12/1/2020

Personnel Executing the Validation:
Your signature indicates that you have read and understand this protocol.

Printed Name	Signature	Tasks Performed
(b) (6)		Analyst 1
		Analyst 2

Purpose:
This validation will demonstrate that the DY10 Solid Intermediate Microbe Enumeration method can quantify the amount of DY10 (*Ruminococcus bovis)* in solid intermediate samples such as: Preservation by Vaporization (PBV), Milled Preservation by Vaporization MPBV), and Lipid Encapsulate. The following parameters will be tested in this validation:

- Repeatability – closeness of results obtained on the same sample when assayed multiple times by the same person with the same reagents and equipment.
- Robustness – reliability of the method to withstand small variations such as different technicians and reagent preparations.
- Linearity – the assay produces reliable results over a range of concentrations.

Background:

DY10 (*Ruminococcus bovis)* solid intermediates are produced by freeze drying the Preservation Mixture to product PBV material, which is milled to product MPBV, then coated with wax to produce the lipid encapsulate. Samples from any of the three steps may be tested with this method. The lipid encapsulate is used as an active ingredient in the finished product. The microbe enumeration assay was developed by Native Microbials.

The growth conditions (media, time, and temperature) for each organism were selected based on standard lab practices for these organisms, development studies, and similar approved methods. All reagents are known to be stable for the duration of the validation activities.

Method Overview:

(b) (4)

Sample Preparation:

(b) (4)

Primary Dilution Preparation

Sample #	Sample Type	Sample Lot Number/ID	Approximate Viability
1	Lot A, normal concentration	(b) (4)	~5E8 CFU/g
2	Lot A, 5x lower concentration		~5E8 CFU/g
3	Lot A, 10x lower concentration		~5E8 CFU/g
4	Lot B, normal concentration		~1E10 CFU/g

Validation Approach:

Version 1 of the DY10 Solid Intermediate Microbe Enumeration method will be followed. The method is located here: (b) (6) (b) (6)

Sample 1 will be assayed three times by analyst 1 to demonstrate repeatability of the assay.

Samples 2 – 4 will be assayed one time by analyst 1.

A second analyst will assay samples 1 – 4.

Each analyst will use different batches of reagents and plates.

The closeness of results between analysts will be assessed to determine the robustness of the assay. Graphs of samples 1-3 will be generated to demonstrate assay linearity.

All equipment calibrations are recorded in lab documentation. Raw data will be recorded directly in the protocol.

Data Analysis:

The calculation for converting the raw colony numbers to the CFU/ml is listed in the method. The CV and Standard Deviation calculations are also listed in the method.

Acceptance Criteria:

- The assay yields comparable results when the same sample is assayed multiple times by one analyst (repeatability).
- The assay is robust when the same sample is assayed by different personnel with different reagents.
- The assay is linear.
- Coefficient of Variation (CV%) is +/- 75% for results on the same sample.

Summary and Conclusions:

A summary report will be prepared based on the validation results. Post-approval of the executed protocol and the summary report will occur simultaneously. The summary will include the following information:

- Changes to the original protocol
- Deviations from the protocol
- Statistical analysis of the data
- Conclusions developed from the data, including if the acceptance criteria were met
- Statement as to the method validation status
- Location of all raw data (if not recorded in the protocol).

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Data Collection – Analyst 1 Name_[redacted]_____

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-1	[redacted]			[redacted] 23NOV 2020
1-2	[redacted]			[redacted] 23NOV 2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-3	[redacted]	[redacted]	[redacted]	[redacted] (b) (6) 23NOV2020
2	[redacted]	[redacted]	[redacted]	[redacted] (b) (6) 23NOV2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	[redacted]	[redacted]	[redacted]	[redacted] (b) (6) 23NOV2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	[redacted]	[redacted]	[redacted]	[redacted] (b) (6) 23NOV2020

CFU Results

Sample			Initial/Date		Sample			Initial/Date
1-1	Final Result (CFU/~~mL~~)	[redacted]	[redacted]		1-3	Final Result (CFU/~~mL~~)	[redacted]	[redacted]
	Standard Deviation	[redacted]	[redacted]			Standard Deviation	[redacted]	[redacted]
1-2	Final Result (CFU/~~mL~~)	[redacted]	[redacted]		2	Final Result (CFU/~~mL~~)	[redacted]	[redacted]
	Standard Deviation	[redacted]	[redacted]			Standard Deviation	[redacted]	[redacted]

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CFU Results

Sample			Initial/Date		Sample			Initial/Date
3	Final Result (CFU/~~mL~~)	[redacted]	2/3/2020		4	Final Result (CFU/~~mL~~)	[redacted]	2/3/2020
	Standard Deviation	[redacted]	2/3/2020			Standard Deviation	[redacted]	2/3/2020

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Data Collection – Analyst 2 Name [redacted]

Sample ID	Replicate	Dilution	Colonies	Initial/Date	Sample ID	Replicate	Dilution	Colonies	Initial/Date
1	[redacted]			[redacted] 18Nov20	2	[redacted]			[redacted] 18Nov20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3			(b) (4)	(b) (6) 18Nov20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4			(b) (4)	(b) (6) 18Nov20

CFU Results

Sample			Initial/Date	Sample			Initial/Date
1	Final Result ~~(CFU/mL)~~ CFU/g	[redacted]	23Nov20	3	Final Result (CFU/mL) CFU/g	[redacted]	23Nov20
	Standard Deviation		23Nov20		Standard Deviation		23Nov20
2	Final Result ~~(CFU/mL)~~ CFU/g		23Nov20	4	Final Result ~~(CFU/mL)~~ CFU/g		23Nov20
	Standard Deviation		23Nov20		Standard Deviation		23Nov20

[redacted]

[redacted]

Method Validation Summary Report

Method

Dairy-10 Solid Intermediate Microbe Enumeration, V1

Objective

The objective of this validation was to demonstrate that the DY10 Solid Intermediate Microbe Enumeration method can quantify the amount of DY10 (*Ruminococcus bovis* ASCUSDY10) in solid forms such as the *Ruminococcus bovis* ASCUSDY10 (DY10) Fat Encapsulate final product. The method was evaluated for repeatability, robustness, and linearity.

Repeatability was assessed through the closeness of results obtained on the same sample (b) (4)) when assayed multiple times by the same person with the same reagents and equipment.

Robustness was assessed through the closeness of results obtained on the same set of samples ((b) (4) and (b) (4)) across multiple analysts and reagent preparations.

Linearity was assessed by enumerating the same sample at a concentration of 20% and 10% of the original sample ((b) (4)).

Results

Repeatability

The average of samples 1-1, 1-2, and 1-3 is 5.77E+08 CFU/g with a standard deviation of 2.99E+07 CFU/g. The coefficient of variation from these samples is 5%. The low CV resulting from repeated measurements of the same sample demonstrates the repeatability of the assay.

Table 1: Summary table of DY10 solid enumeration method validation results

		Average CFU/g	STDEV	CV
Analyst 1	Sample 1-1	(b) (4)		
	Sample 1-2			
	Sample 1-3			
	Sample 2			
	Sample 3			
	Sample 4			
Analyst 2	Sample 1			
	Sample 2			
	Sample 3			
	Sample 4			

Robustness

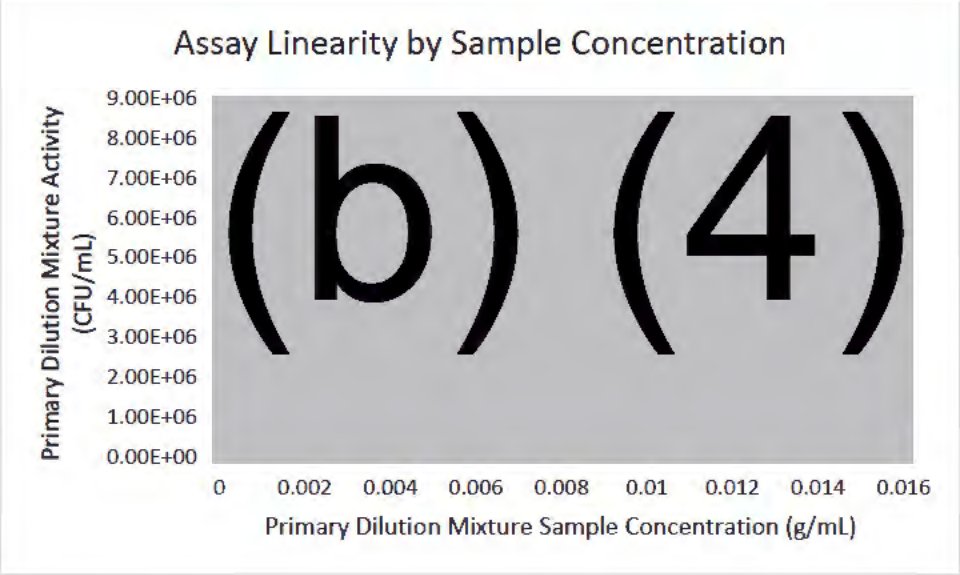
Samples 1-4 were enumerated by two independent analysts. All measurements yielded a CV less than 30% within an analyst's measurements and a CV less than 20% for combined measurements of both analysts, demonstrating that the assay is robust to different analysts and reagent preparations.

Table 2: Summary of Repeatability, Linearity, and Robustness

Repeatability			
Average Sample 1	(b) (4)		
5.77E+08			
Linearity			
$R^2 = 0.93$			
Robustness across analysts			
	Average	STDEV	CV
Sample 1	(b) (4)		
Sample 2			
Sample 3			
Sample 4			

Linearity

Sample 2 was prepared by diluting Sample 1 to 20%, and Sample 3 was prepared by diluting Sample 1 to 10% in the primary dilution mixture. The activity (CFU/mL) of the resulting primary dilution mixtures was plotted against the sample concentration (g/mL). The resulting linear regression had an R^2 value of 0.93, demonstrating linearity between the two parameters.



Conclusion

The Dairy-10 Solid Intermediate Microbe Enumeration assay is valid, demonstrated by the repeatability, robustness, and linearity of the assay. The protocol was executed as written with no deviations or changes during execution.

Raw data and analysis can be found on the compan (b) (6)
(b) (6)

Approval

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: (b) (6) ACBDDAD433BF491...	12/1/2020
(b) (6) Quality	(b) (6)	12/1/2020

Method

Title	Dairy-10 Solid Intermediate Microbe Enumeration
Version	01
Effective Date	13Nov2020
Author	(b) (6)
Approver (Signature & Date)	DocuSigned by: (b) (6) ACBDDAD433BF491... 11/10/2020 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Ruminococcus bovis* in Dairy-10 solid intermediates in samples from:

- Preservation by Vaporization (PBV) or milled PBV (mPBV) intermediates
- Lipid Encapsulated intermediates

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with liquid nitrogen and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

1000 µL pipette tips, sterile, anaerobic
200 µL pipette tips, sterile, anaerobic
20 µL pipette tips, sterile, anaerobic
96-well (8x12 well) 200 µL plate, sterile, anaerobic
Reagent reservoir, sterile, anaerobic
1.5 mL microcentrifuge tubes, sterile, anaerobic
Liquid Nitrogen
10% Bleach
>70% Ethanol or Isopropanol

Equipment

Autoclave
Laboratory Vortexer
Mortar and Pestle
Anaerobic Chamber
Dissection microscope or magnifying glass
1000 µL Pipette
200 µL Pipette
200 µL Multi-channel Pipette
20 µL Multi-channel Pipette

Media & Reagents

RCM plates

Anaerobic Phosphate Buffered Saline (PBS)

(recipes can be found here:

(b) (6)

Method

1. De-encapsulation of DY10 Lipid Encapsulate

(b) (4)

2. Prepare the Primary Dilution Mix

(b) (4)

3. DY10 Solid Intermediate Anaerobic Plating

(b) (4)

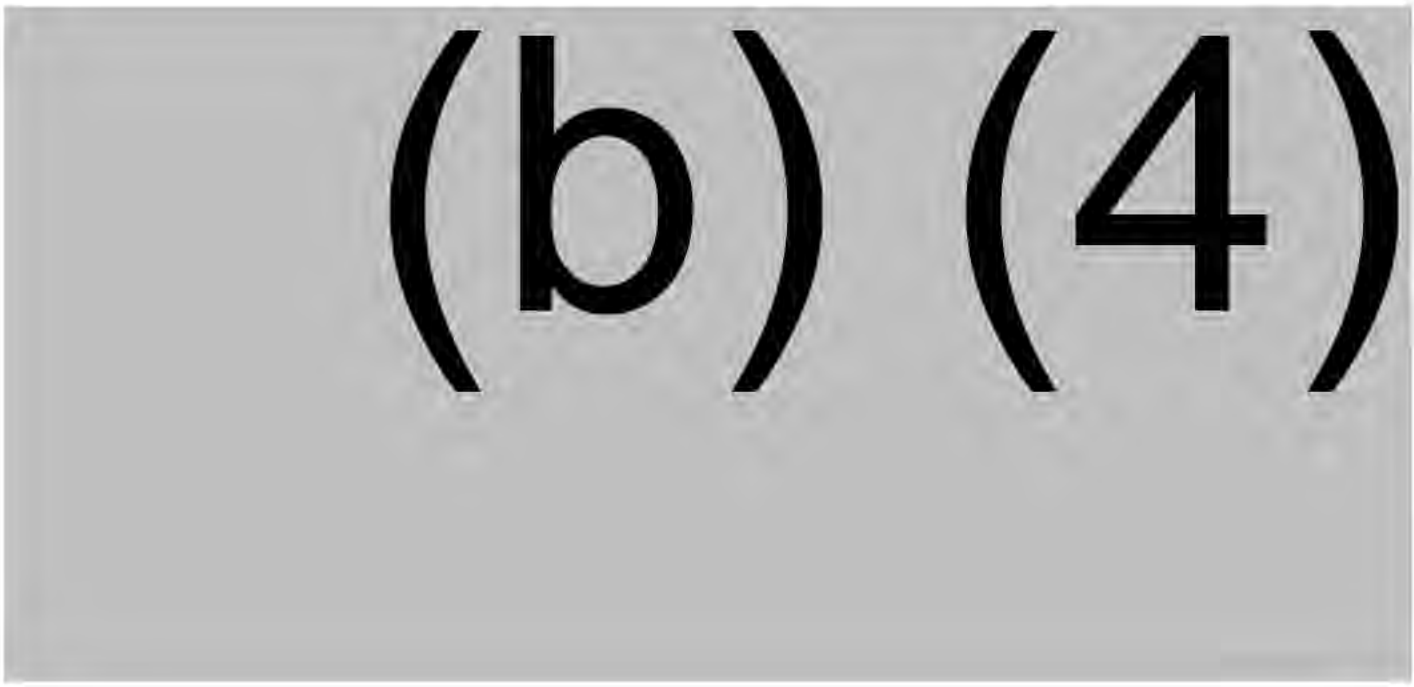
(b) (4)

4. Spot Plating

(b) (4)

5. Enumeration and Colony Forming Unit Determination

(b) (4)



ASCUS BIOSCIENCES

Method

Title	**Moisture Analysis**
Version	**01**
Effective Date	**15Dec2019**
Author	(b) (6)
Approver (Signature & Date)	DocuSigned by: (b) (6) D1605F1B4C3E49A... 12/3/2019 **Martin Mayhew – VP – Process Development & Manufacturing**

Scope

This method is used to determine the moisture content of solid samples such as Galaxis 100, Altius 5, DY20 SDP, and DY21 POE.

Safety

Wear safety goggles, lab coat, and gloves when handling samples.
Use caution when removing the sample as the sample, chamber, and draft shield may be extremely hot.

Materials

None

Equipment

(b) (4) Moisture Analyzer (multiple models may be used)

Media and Reagents

None

Method

(b) (4)

(b) (4)

Reasons for Revision

(b) (4)

Method

Title	Dairy-10 Liquid Intermediate Microbe Enumeration
Version	01
Effective Date	11Oct2020
Author	(b) (6)
Approver (Signature & Date)	Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Ruminococcus bovis* in Dairy-10 liquid intermediates in samples from:

- End of Fermentation
- Cell Concentrate
- Preservation Mixture

Safety

Consult the Safety Data Sheet for all reagents prior to handling.

Materials

1000 µL pipette tips, sterile, anaerobic
200 µL pipette tips, sterile, anaerobic
20 µL pipette tips, sterile, anaerobic
96-well (8x12 well) 200 µL plate, sterile, anaerobic
Reagent reservoir, sterile, anaerobic
1.5 mL microcentrifuge tubes, sterile, anaerobic

Equipment

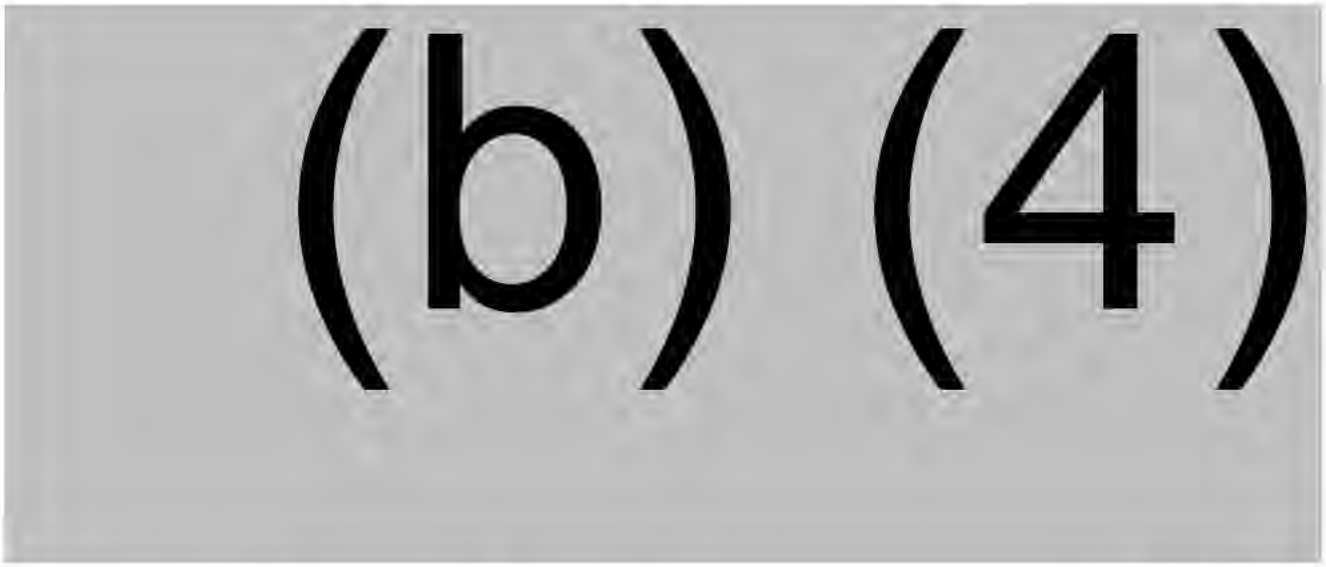
Autoclave
Laboratory Vortexer
Anaerobic Chamber
Dissection microscope or magnifying glass
1000 µL Pipette
200 µL Pipette
200 µL Multi-channel Pipette
20 µL Multi-channel Pipette

Media & Reagents

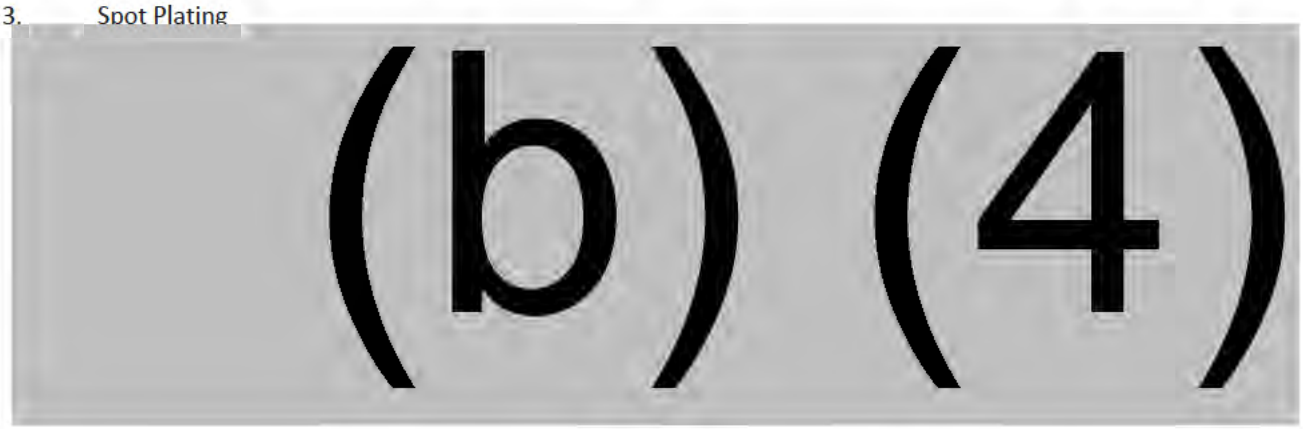
RCM plates
Anaerobic Phosphate Buffered Saline (PBS)
(b) (6)

Method

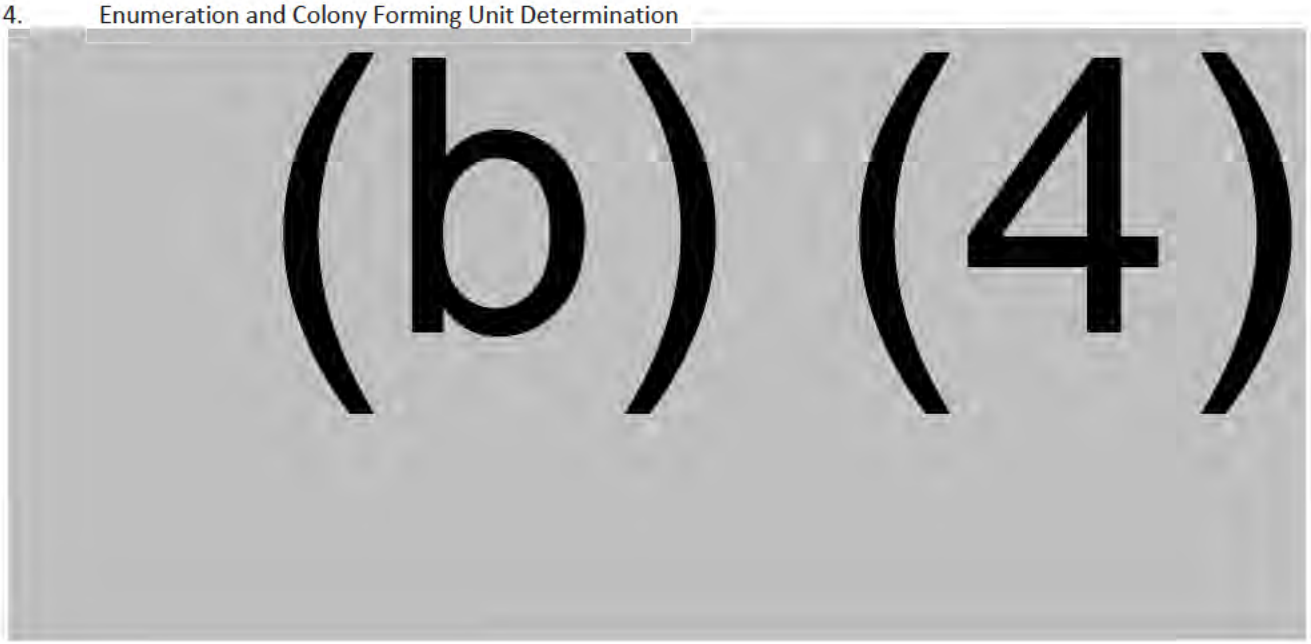
(b) (4)

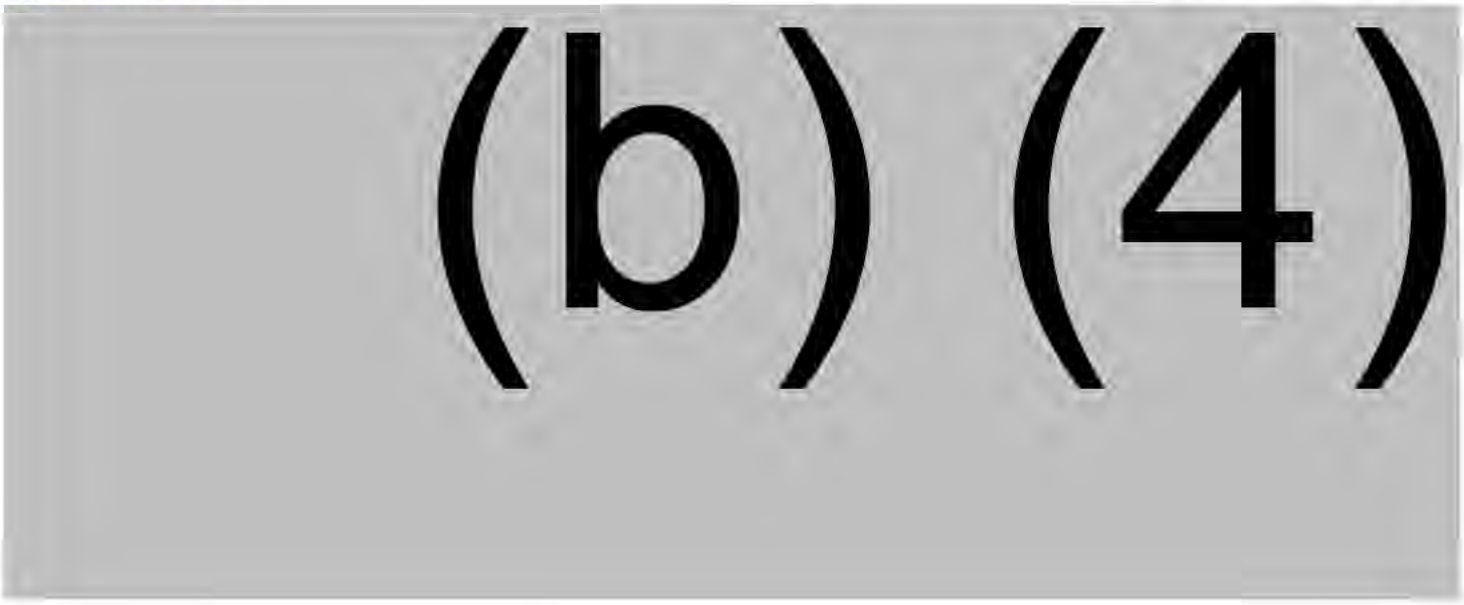


3. Spot Plating



4. Enumeration and Colony Forming Unit Determination





native

Method Validation Protocol, Version 1

Method Title and Versions

Title	DY10 Liquid Intermediate Microbe Enumeration
Version	01 Draft

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b) (6)	9/23/2020
(b) (6) – Quality		9/23/2020

Post Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b) (6)	10/1/2020
(b) (6) – Quality		10/1/2020

Personnel Executing the Validation:
Your signature indicates that you have read and understand this protocol.

Printed Name	Signature	Tasks Performed
(b) (6)	(b) (6)	Analyst 1
		Analyst 2

Purpose:
This validation will demonstrate that the DY10 Liquid Intermediate Microbe Enumeration method can quantify the amount of DY10 (*Ruminococcus bovis)* in liquid intermediates, such as End of Fermentation, Cell Concentrate, and Preservation Mixture samples. The following parameters will be tested in this validation:

- Repeatability – closeness of results obtained on the same sample when assayed multiple times by the same person with the same reagents and equipment.
- Robustness – reliability of the method to withstand small variations such as different technicians and reagent preparations.
- Linearity – the assay produces reliable results over a range of concentrations.

Background:

DY10 (*Ruminococcus bovis)* liquid intermediates are produced during the fermentation process of the organism. Samples may be tested at the end of the fermentation, after concentration of cells, after the addition of the preservation buffer. The Preservation Mixture is further processed into a powder that will be used in the final product. The microbe enumeration assay was developed by Native Microbials.

The growth conditions (media, time, and temperature) for each organism were selected based on standard lab practices for these organisms, development studies, and similar approved methods. All reagents are known to be stable for the duration of the validation activities.

Method Overview:

(b) (4)

Sample Preparation:

(b) (4)

Primary Dilution Preparation

Sample #	Sample Type (EoF or CC)	Sample Lot Number/ID	Approximate Viability
1	EoF normal concentration	(b) (4)	~2E9
2	EoF 5x lower concentration		~4E8
3	EoF 10x lower concentration		~2E8
4	CC normal concentration		~5E10
5	PM normal concentration		~2E10

Validation Approach:
The draft version of the DY10 Liquid Intermediate Microbe Enumeration method will be followed. The method is included at the end of this document.

Sample 1 will be assayed three times by analyst 1 to demonstrate repeatability of the assay.

Samples 2 – 5 will be assayed one time by analyst 1.

A second analyst will assay samples 1 – 5.

Each analyst will use different batches of reagents and plates.

The closeness of results between analysts will be assessed to determine the robustness of the assay. Graphs of the EoF data will be generated to demonstrate assay linearity.

All equipment calibrations are recorded in lab documentation. Raw data will be recorded directly in the protocol.

Data Analysis:
The calculation for converting the raw colony numbers to the CFU/ml is listed in the method. The CV and Standard Deviation calculations are also listed in the method.

Acceptance Criteria:

- The assay yields comparable results when the same sample is assayed multiple times by one analyst (repeatability).
- The assay is robust when the same sample is assayed by different personnel with different reagents.
- The assay is linear.
- Coefficient of Variation (CV%) is +/- 75% for results on the same sample.

Summary and Conclusions:

A summary report will be prepared based on the validation results. Post-approval of the executed protocol and the summary report will occur simultaneously. The summary will include the following information:

- Changes to the original protocol
- Deviations from the protocol
- Statistical analysis of the data
- Conclusions developed from the data, including if the acceptance criteria were met
- Statement as to the method validation status
- Location of all raw data (if not recorded in the protocol).

native

Data Collection – Analyst 1 Name (b) (6)

Sample ID	Replicate	Dilution	Colonies	Initial/Date	Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-1	(b) (4)			(b) (6) 28SEP2020	1-2	(b) (4)			(b) (6) 28SEP2020

native

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-3	(b) (4)			(b) (6) 28SEP2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	(b) (4)			(b) (6) 28SEP2020

native

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	(b) (4)			(b) (6) 28SEP2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	(b) (4)			(b) (6) 28SEP2020

native

Sample ID	Replicate	Dilution	Colonies	Initial/Date
5	(b) (4)			(b) (6) 28SEP2020

native

CFU Results

Sample			Initial/Date		Sample			Initial/Date
1-1	Final Result (CFU/mL)	(b) (4)			1-3	Final Result (CFU/mL)	(b) (4)	
	Standard Deviation	(b) (6), (b) (4)				Standard Deviation	(b) (6), (b) (4)	
1-2	Final Result (CFU/mL)				2	Final Result (CFU/mL)		
	Standard Deviation					Standard Deviation		

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CFU Results

Sample			Initial/Date		Sample			Initial/Date
3	Final Result (CFU/mL)	(b) (4)			5	Final Result (CFU/mL)	(b) (6), (b) (4)	
	Standard Deviation	(b) (6), (b) (4)				Standard Deviation		
4	Final Result (CFU/mL)							
	Standard Deviation							

native

Data Collection – Analyst 2 Nam(b) (6)

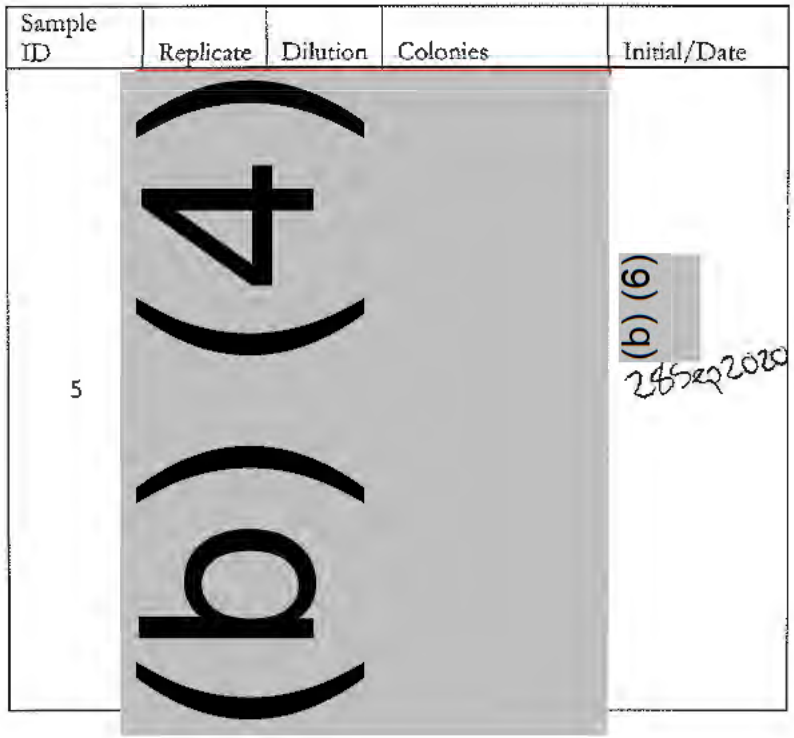
Sample ID	Replicate	Dilution	Colonies	Initial/Date
1	(b) (4)			(b) (6) 28Sep2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	(b) (4)			(b) (6) 28Sep2020

native

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	(b) (4)			(b) (6) 28Sep2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	(b) (4)			(b) (6) 28Sep2020



Sample ID	Replicate	Dilution	Colonies	Initial/Date
5				(b) (6) 2/5/2020

native

CFU Results

Sample			Initial/Date		Sample			Initial/Date
1	(b) (4), (b) (6), (b) (4)		28Sep2020		3	(b) (4), (b) (6), (b) (4)		28Sep2020
			28Sep2020					28Sep2020
2			28Sep2020		4			28Sep2020
			28Sep2020					28Sep2020

native

CFU Results

Sample			Initial/Date		Sample			Initial/Date
5	(b) (6), (b) (4)		2/5/2020					
			2/5/2020					

native MICROBIALS

Draft Method

Title	Dairy-10 Liquid Intermediate Microbe Enumeration
Version	01
Effective Date	Draft
Author	(b) (6)
Approver (Signature & Date)	(b) (6) 10/1/2020 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Ruminococcus bovis* in Dairy-10 liquid intermediates in samples from:

- End of Fermentation
- Cell Concentrate
- Preservation Mixture

Safety

Consult the Safety Data Sheet for all reagents prior to handling.

Materials

1000 µL pipette tips, sterile, anaerobic
200 µL pipette tips, sterile, anaerobic
20 µL pipette tips, sterile, anaerobic
96-well (8x12 well) 200 µL plate, sterile, anaerobic
Reagent reservoir, sterile, anaerobic
1.5 mL microcentrifuge tubes, sterile, anaerobic

Equipment

Autoclave
Laboratory Vortexer
Anaerobic Chamber
Dissection microscope or magnifying glass
1000 µL Pipette
200 µL Pipette
200 µL Multi-channel Pipette
20 µL Multi-channel Pipette

Media & Reagents

RCM plates
Anaerobic Phosphate Buffered Saline (PBS)
(b) (6)

Method

(b) (4)

1. (b) (4)
2. Sample Dilution

(b) (4)

3. Spot Plating

(b) (4)

4. Enumeration and Colony Forming Unit Determination

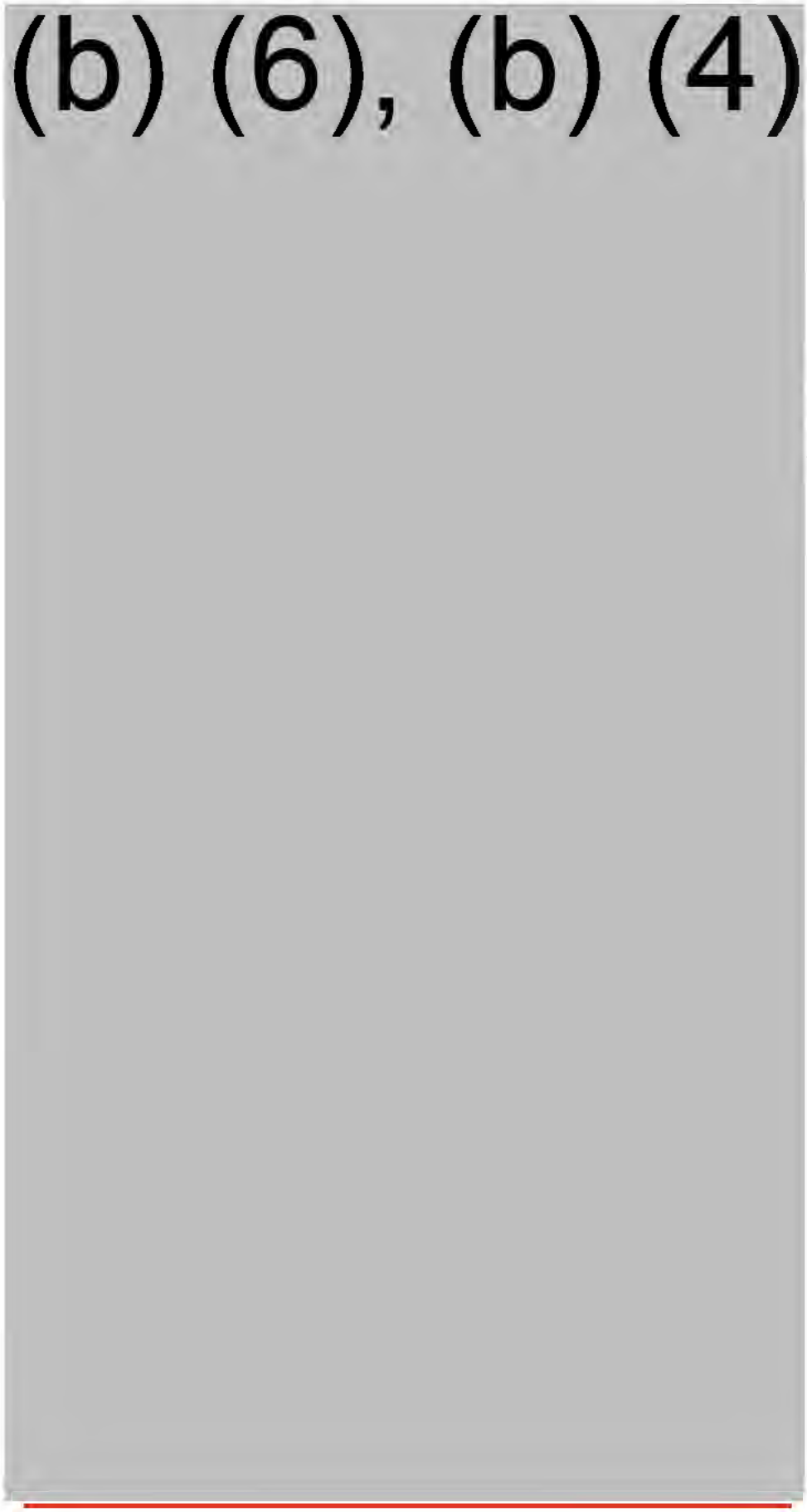
(b) (4)

(b) (4)

(b) (6), (b) (4)

Date 24Sep2020

LM002



(b) (6), (b) (4)

Date: 24Sep2020

LMO34

(b) (6), (b) (4)

Date: 24 Sep 2020

LM034

Validation Summary Report

Dairy-10 Liquid Intermediate Microbe Enumeration, V1

Objective

The objective of this validation was to demonstrate that the DY10 Liquid Intermediate Microbe Enumeration method can quantify the amount of DY10 (*Ruminococcus bovis*) in liquid intermediates such as End of Fermentation (EOF), Cell Concentrate (CC), and Preservation Mixture (PM) samples. The method was evaluated for repeatability, robustness, and linearity.

Repeatability was assessed through the closeness of results obtained on the same sample (b) (4) when assayed multiple times by the same person with the same reagents and equipment.

Robustness was assessed through the closeness of results obtained on the same set of samples (b) (4) across multiple analysts and reagent preparations.

Linearity was assessed by enumerating the same sample at a concentration of 20% and 10% of the original sample (b) (4)).

Results

Repeatability

The average of samples 1-1, 1-2, and 1-3 is 3.67E+09 CFU/mL with a standard deviation of 2.23E+08 CFU/mL. The coefficient of variation from these samples is 6%. The low CV resulting from repeated measurements of the same sample demonstrates the repeatability of the assay.

Table 1: Summary table of DY10 liquid enumeration method validation results

		Average CFU/mL	STDEV	CV
Analyst 1	Sample 1-1	(b) (4)		
	Sample 1-2			
	Sample 1-3			
	Sample 2			
	Sample 3			
	Sample 4			
	Sample 5			
Analyst 2	Sample 1			
	Sample 2			
	Sample 3			
	Sample 4			
	Sample 5			

Robustness

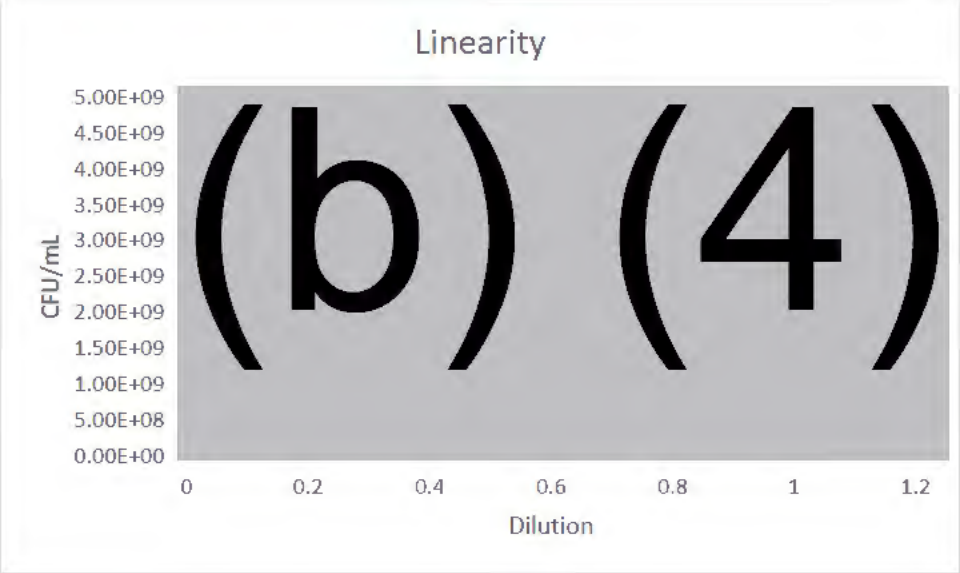
Samples 1-5 were enumerated by an independent analyst. All measurements yielded a CV less than 22% for both analysts, and the data measured for samples across analysts yielded CVs less than 22%, suggesting that the measurement is robust to different analysts and reagent preparations.

Table 2: Summary of Repeatability, Linearity, and Robustness

Repeatability		
Average Sample 1	STDEV	CV
3.67E+09	(b) (4)	
Linearity		
R^2 = 0.973		
Robustness across analysts		
	Average	STDEV	CV
Sample 1	(b) (4)		
Sample 2			
Sample 3			
Sample 4			
Sample 5			

Linearity

Sample 2 was prepared by diluting Sample 1 to 20%, and Sample 3 was prepared by diluting Sample 1 to 10%. All replicates were plotted vs. 1 (Sample 1), 0.2 (Sample 2), or 0.1 (Sample 3). The resulting linear regression had an R^2 value of 0.973, suggesting strong linearity between the dilution and measured CFU/mL.



Conclusion

The protocol was executed as written with no deviations or changes during execution. Repeatability, robustness, and linearity of the assay were demonstrated.

All acceptance criteria were met and this assay is suitable for use on development and commercial product.

Raw data and analysis can be found on the company (b) (6) (b) (6)

Approvals

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	(b) (6) 10/1/2020
(b) (6) Quality	(b) (6) 10/1/2020

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Freeze-dried Powder
Batch Number	(b) (4)
Date of Manufacture	04Nov2020
Expiration Date	N/A
Retest Date	04Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6) 12/28/2020

Quality

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Freeze-dried Powder
Batch Number	(b) (4)
Date of Manufacture	04Nov2020
Expiration Date	N/A
Retest Date	04Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6) 12/28/2020

Quality

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Freeze-dried Powder
Batch Number	(b) (4)
Date of Manufacture	04Nov2020
Expiration Date	N/A
Retest Date	04Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6) 12/28/2020

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Fat Encapsulated Product
Batch Number	(b) (4)
Date of Manufacture	01Dec2020
Expiration Date	N/A
Retest Date	01Dec2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6) 12/28/2020

Quality

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Fat Encapsulated Product
Batch Number	(b) (4)
Date of Manufacture	03Dec2020
Expiration Date	N/A
Retest Date	03Dec2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6) 12/28/2020

Quality

Product Certificate of Analysis

Product Name	*Ruminococcus bovis* ASCUSDY10 Fat Encapsulated Product
Batch Number	(b) (4)
Date of Manufacture	30Nov2020
Expiration Date	N/A
Retest Date	30Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count	(b) (4)	
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

(b) (6)

12/28/2020

Quality

Analysis of *Ruminococcus bovis* ASCUSDY10 (DY10) POE for Heavy Metals & Microbial Contamination

Approvers:

(b) (6) 12/21/2020

hew — Date

Vice President – Product Development & Manufacturing

(b) (6) 12/18/2020

Date

Quality

(b) (6) 12/18/2020

Kevin Korth — Date

Regulatory

Prepared by
Native Microbials, Inc
San Diego, CA

December 2020

Analysis of *Ruminococcus bovis* ASCUSDY10 POE for Heavy Metals & Microbial Contamination

Three lots of *Ruminococcus bovis* ASCUSDY10 POE were sent for heavy metal and microbial contamination analysis at (b) (6), (b) (4) (Note: *R. bovis* ASCUSDY10 is listed on certificate of analysis as Dairy-10 Fat Encapsulate which was internal name used by Native Microbials, Inc.)

The ICP-MS/AOAC 2015.01 method was used for the heavy metal analysis of the samples and results are summarized in the following table.

Table 1. Heavy Metal Analysis of Three Lots of *Ruminococcus bovis* ASCUSDY10 POE

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.004	0.0008	0.001	0.001
(b) (4)	0.012	ND	0.006	ND
	ND	ND	0.007	ND
	0.011	ND	0.003	ND

ND – None Detected

The methods used for analysis were AOAC 2018.13 for Coliforms/E. coli, AOAC 2013.01 for Salmonella, and AOAC 2013.10 for Listeria. Results are summarized in the following table.

Table 2. Microbial Contamination Testing for *Ruminococcus bovis* ASCUSDY10 POE

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
(b) (4)	<10	<10	Negative	Negative
	<10	<10	Negative	Negative
	<10	<10	Negative	Negative

R. bovis ASCUSDY10 POE is intended to be fed as part of the product mixed in a grain premix then further diluted in a total mixed ration or grain supplement. Given the low inclusion rate in the grain mix (5 g/cow/day) and further dilution in the total mixed ration, no heavy metal specification is needed. However, all lots will be tested for microbial contamination at the end of the production of *R. bovis* ASCUSDY10 POE.

Attachment 1. Certificate of Analysis – Heavy Metal Analysis (b) (4) Sample No. 1065821)

(b) (4)

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No. (b) (4)
Sample No. 1065821

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.012
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.006

Reported by
(b) (6), (b) (4)
December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (6), (b) (4)

Attachment 2. Certificate of Analysis – Heavy Metal Analysis (b) (4) Sample No. 1065822)

(b) (4)

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No (b) (4)
Sample No. 1065822

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	None detected
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.007

Reported by
(b) (4), (b) (6)

December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (4), (b) (6)

Attachment 3. Certificate of Analysis –Heavy Metal Analysis (b) (4) Sample No. 1065823)

(b) (4)

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No. (b) (4)
Sample No. 1065823

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.011
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.003

Reported by
(b) (4)

December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (6), (b) (4)

Attachment 4. Certificate of Analysis – Microbial Contamination Testing (b) (4) Sample No. 1065821)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No (b) (4)
Sample No. 1065821

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (6), (b) (4)

(b) (6), (b) (4)

Attachment 5. Certificate of Analysis – Microbial Contamination Testing (b) (4) Sample No. 1065822)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No. (b) (4)
Sample No. 1065822

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (6), (b) (4)

(b) (6), (b) (4)

Attachment 6. Certificate of Analysis – Microbial Contamination Testing (b) (4) Sample No. 1065823)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No. (b) (4)
Sample No. 1065823

SAMPLE INFORMATION

Description	Dairy-10 Fat Encapsulate
Lot Number	(b) (4)
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (6), (b) (4)

(b) (6), (b) (4)

DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate 5°C Stability Report

Purpose

The purpose of this report is to present the results and analysis of the real time stability study of DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate lots 1801.2034, 1801.2036, and 1801.2038 stored at 5°C to support the prediction of product stability at 2-10°C.

Results

Samples were placed at 5°C and analyzed monthly for viable cell count according to the approved Stability Protocol for DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate. See Table 1 below for test timepoints.

Table 1 – Tests and timepoints.

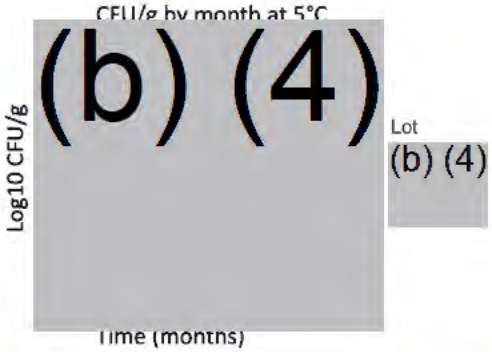
Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY10 Solid Intermediate Microbe Enumeration method	(b) (4)						

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	(b) (4)		
0	4.77E+09	2.46E+09	4.00E+09
1	(b) (4)		
2			
3			
6			
9			
12			

Figure 1 – CFU/g by month



Log_{10} CFU/g measurements are plotted, with the minimum specification represented as zero on the y-axis. Shaded area represents the 95% confidence interval.

Conclusion

Real time stability data collected for 12 months at 5°C demonstrates that all 3 lots of DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate remain above the minimum specification for the duration tested.

Data Availability

All data is retained and available on the company (b) (6)

(b) (6)

Stability Protocol

native

nativemicrobials.com

Stability Protocol Title:	DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate 5°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY10 Solid Intermediate Microbe Enumeration method	(b) (4)						

Protocol Approvals:

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	(b) (6)	12/2/2020
Howard Green Regulatory		12/1/2020
(b) (6)		12/2/2020

DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate 25°C Stability Report

Purpose

The purpose of this report is to present the results and analysis of the real time stability study of DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate lots (b) (4) and (b) (4) stored at 25°C to support the prediction of product stability at 2-10°C.

Results

Samples were placed at 25°C and analyzed monthly for viable cell count according to the approved Stability Protocol for DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate. See Table 1 below for test timepoints.

Table 1 – Tests and timepoints.

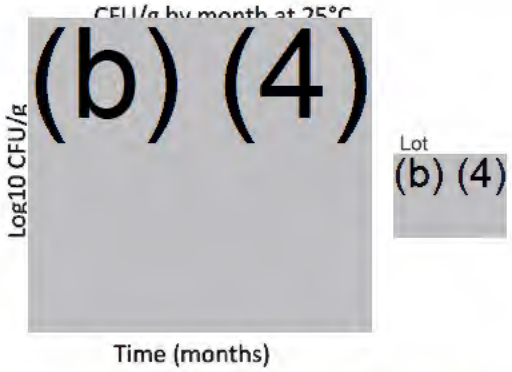
Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY10 Solid Intermediate Microbe Enumeration method	(b) (4)						

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	(b) (4)		
0	4.77E+09	2.46E+09	4.00E+09
1	(b) (4)		
2			
3			
6			
9			
12			

Figure 1 – CFU/g by month



Log_{10} CFU/g measurements are plotted, with the minimum specification represented as zero on the y-axis. Shaded area represents the 95% confidence interval.

Conclusion

Real time stability data collected for 12 months at 25°C demonstrates that all 3 lots of DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate remain above the minimum specification for the duration tested.

Data Availability

All data is retained and available on the company (b) (6)

(b) (6)

Stability Protocol

native

nativemicrobials.com

Stability Protocol Title:	DY10 *Ruminococcus bovis* ASCUSDY10 Fat Encapsulate 25°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY10 Solid Intermediate Microbe Enumeration method	(b) (4)						

Protocol Approvals:

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	(b) (6)	12/2/2020
Howard Green Regulatory		12/1/2020
	(b) (6)	12/2/2020

Product Certificate of Analysis

Product Name	Dairy-10 Preservation Mixture (DY10 PM)
Batch Number	(b) (4)
Date of Manufacture	18Jan2022
Expiration Date	N/A
Retest Date	18Jan2023
Storage Conditions	< -40 °C

Analytical Property	Specification	Result
DY10 Liquid Intermediate Microbe Enumeration	(b) (4)	
Dry Solids		
Coliform		
E. coli		
Salmonella		
Listeria		
Botulinum Toxin		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6)

2/1/2022

Quality Manager

(b) (4)

Certificate of Analysis

January 28, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 530846
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 Cryo-pellets
Lot Number	(b) (4)
Received	January 25, 2022

ANALYTICAL RESULTS

Analysis Date January 25, 2022 to January 28, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	AOAC 2018.13
	E. coli		AOAC 2018.13
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

(b) (6), (b) (4)

(b) (6), (b) (4)

Name: Native Microbials, Inc.
Customer: Martin Mayhew
Address: 10255 Science Center Dr., Suite C2
San Diego, CA
92121
USA
877-696-8945 x 731

Order ID: (b) (4)
Report ID:
Date Received: 1/21/2022 10:13:13
Reported: 1/28/2022 17:08:27
P.O. #: N/A
Page: 1 of 1

Report of Results

(b) (4) **Analysis Date:** 2022/01/21 **Receiving Temperature:** 2.6C **Sample Condition:** Okay

Description: Product: DY10 Preservation Mixture Lot: (b) (4)

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

(b) (6), (b) (4)

(b) (4)

Certificate of Analysis

January 26, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 530845
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 Cryo-pellets
Lot Number	(b) (4)
PO#	
Received	January 25, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	January 25, 2022 to January 26, 2022

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

ND = None Detected

(b) (6), (b) (4)

January 26, 2022

(b) (6), (b) (4)

Product Certificate of Analysis

Product Name	Dairy-10 Preservation Mixture (DY10 PM)
Batch Number	(b) (4)
Date of Manufacture	03May2022
Expiration Date	N/A
Retest Date	03May2023
Storage Conditions	< -40 °C

Analytical Property	Specification	Result
DY10 Liquid Intermediate Microbe Enumeration	(b) (4)	
Dry Solids		
Coliform		
E. coli		
Salmonella		
Listeria		
Botulinum Toxin		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6) 5/13/2022

Quality Manager

(b) (4)

Certificate of Analysis

May 13, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 534101
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 Preservation Mixture
Lot Number	(b) (4)
Received	May 10, 2022

ANALYTICAL RESULTS

Analysis Date May 10, 2022 to May 13, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	FDA BAM - ECC Agar
	E. coli		FDA BAM - ECC Agar
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

(b) (6), (b) (4)

(b) (6), (b) (4)

Name: Native Microbials, Inc.
Customer: Martin Mayhew
Address: 10255 Science Center Dr., Suite C2
San Diego, CA
92121
USA
877-696-8945 x 731

Order ID: (b) (4)
Report ID:
Date Received: 5/6/2022 09:34:26
Reported: 5/13/2022 12:29:32
P.O. #: ALW COM DY
Page: **1 of 1**

Report of Results

(b) (4) **Analysis Date:** 2022/05/06 **Receiving Temperature:** 2.5C **Sample Condition:** Okay

Description: NM042122F5

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

(b) (6), (b) (4)

(b) (4)

Certificate of Analysis

May 12, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 534102
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 Preservation Mixture
Lot Number	(b) (4)
PO#	
Received	May 10, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	May 10, 2022 to May 12, 2022

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

ND = None Detected

(b) (4), (b) (6)

May 12, 2022

(b) (6), (b) (4)

Product Certificate of Analysis

Product Name	Dairy-10 Preservation Mixture (DY10 PM)
Batch Number	(b) (4)
Date of Manufacture	28Jun2022
Expiration Date	N/A
Retest Date	28Jun2023
Storage Conditions	< -40 °C

Analytical Property	Specification	Result
DY10 Liquid Intermediate Microbe Enumeration	(b) (4)	
Dry Solids		
Coliform		
E. coli		
Salmonella		
Listeria		
Botulinum Toxin		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6) 7/11/2022

Quality Manager

(b) (4)

Certificate of Analysis

July 11, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 535715
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 PM
Lot Number	(b) (4)
Received	July 06, 2022

ANALYTICAL RESULTS

Analysis Date: July 06, 2022 to July 11, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	FDA BAM - ECC Agar
	E. coli		FDA BAM - ECC Agar
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

(b) (6), (b) (4)

[redacted]

Name: Native Microbials, Inc.
Customer: Martin Mayhew
Address: 10255 Science Center Dr., Suite C2
San Diego, CA
92121
USA
877-696-8945 x 731

Order ID: [redacted]
Report ID: [redacted]
Date Received: 7/1/2022 10:42:11
Reported: 7/13/2022 15:44:53
P.O. #: ALW COM DY
Page: **1 of 1**

Report of Results

[redacted] **Analysis Date:** 2022/07/01 **Receiving Temperature:** 5.4C **Sample Condition:** Okay

Description: [redacted]

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted]

(b) (4)

Certificate of Analysis

July 07, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 535717
Sample No. (b) (4)

SAMPLE INFORMATION

Description	DY10 PM
Lot Number	(b) (4)
PO#	COM DY
Received	July 06, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	July 06, 2022 to July 07, 2022

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

NO = None Detected

(b) (6), (b) (4)

July 07, 2022

(b) (6), (b) (4)

Product Certificate of Analysis

Product Name	Dairy-10 Milled Preservation by Vaporization (DY10 MPBV)
Batch Number	(b) (4)
Date of Manufacture	07Feb2022
Expiration Date	N/A
Retest Date	07Feb2023
Storage Conditions	2-10 °C

Analytical Property	Specification	Result
DY10 Solid Intermediate Microbe Enumeration	(b) (4)	
Moisture Analysis		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6)

2/22/2022

Quality Manager

Product Certificate of Analysis

Product Name	Dairy-10 Milled Preservation by Vaporization (DY10 MPBV)
Batch Number	(b) (4)
Date of Manufacture	17May2022
Expiration Date	N/A
Retest Date	17May2023
Storage Conditions	2-10 °C

Analytical Property	Specification	Result
DY10 Solid Intermediate Microbe Enumeration	(b) (4)	
Moisture Analysis		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6)

5/31/2022

Quality Manager

Product Certificate of Analysis

Product Name	Dairy-10 Milled Preservation by Vaporization (DY10 MPBV)
Batch Number	(b) (4)
Date of Manufacture	11Jul2022
Expiration Date	N/A
Retest Date	11Jul2023
Storage Conditions	2-10 °C

Analytical Property	Specification	Result
DY10 Solid Intermediate Microbe Enumeration	(b) (4)	
Moisture Analysis		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6) 7/29/2022

Quality Manager

Product Certificate of Analysis

Product Name	Lipid Encapsulated *Ruminococcus bovis* ASCUSDY10 (DY10 SOE)
Batch Number	(b) (4)
Date of Manufacture	24Feb2022
Expiration Date	N/A
Retest Date	24Feb2023
Storage Conditions	2-10 °C

Analytical Property	Specification	Result
DY10-POE Microbe Enumeration	(b) (4)	
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6)

3/7/2022

Quality Manager

(b) (4)

Certificate of Analysis

March 07, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 532032
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean Oil Encapsulate
Lot Number	(b) (4)
PO#	
Received	March 03, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	March 03, 2022 to March 07, 2022

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

ND = None Detected

(b) (6), (b) (4)

March 07, 2022

(b) (4), (b) (6)

(b) (4)

Certificate of Analysis

March 07, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 532032
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean Oil Encapsulate
Lot Number	(b) (4)
Received	March 03, 2022

ANALYTICAL RESULTS

Analysis Date March 03, 2022 to March 07, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	AOAC 2018.13
	E. coli		AOAC 2018.13
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

(b) (4), (b) (6)

Product Certificate of Analysis

Product Name	Lipid Encapsulated *Ruminococcus bovis* ASCUSDY10 (DY10 SOE)
Batch Number	(b) (4)
Date of Manufacture	13Jun2022
Expiration Date	N/A
Retest Date	13Jun2023
Storage Conditions	2-10 °C

Analytical Property	Specification	Result
DY10-SOE Microbe Enumeration	(b) (4)	
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)
This batch was manufactured and tested according to Native Microbials standards and meets the registered specifications.

(b) (6)

6/23/2022

Quality Manager

(b) (4)

Certificate of Analysis

July 14, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 535239
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean Encapsulate
Lot Number	(b) (4)
PO#	
Received	June 17, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	June 17, 2022 to July 14, 2022

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

ND = None Detected

(b) (4), (b) (6)

July 14, 2022

(b) (4), (b) (6)

(b) (4)

Certificate of Analysis

June 21, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 535239
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean Encapsulate
Lot Number	(b) (4)
Received	June 17, 2022

ANALYTICAL RESULTS

Analysis Date June 17, 2022 to June 21, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	AOAC 2018.13
	E. coli		AOAC 2018.13
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

(b) (4), (b) (6)

(b) (4)

Certificate of Analysis

August 12, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 536645
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean oil encapsulate
Lot Number	(b) (4)
PO#	
Received	August 05, 2022

ANALYTICAL RESULTS

Analysis	Heavy Metals
Method	ICP-MS
Analysis Date	August 05, 2022 to August 12, 2022

Analyte	LOD / LOQ	Findings
Arsenic	(b) (4)	None Detected
Cadmium		None Detected
Mercury		None Detected
Lead		None Detected

ND = None Detected

(b) (6), (b) (4)

August 12, 2022

(b) (4), (b) (6)

(b) (4)

Certificate of Analysis

August 08, 2022

NATIVE MICROBIALS, INC.
10255 Science Center Dr, Ste C2
San Diego, CA 92121

Order No. 536645
Sample No. (b) (4)

SAMPLE INFORMATION

Description	Dairy-10 Soybean oil encapsulate
Lot Number	(b) (4)
Received	August 05, 2022

ANALYTICAL RESULTS

Analysis Date August 05, 2022 to August 08, 2022

Findings	Analysis	Results	Method
	Coliforms	(b) (4)	FDA BAM - ECC Agar
	E. coli		FDA BAM - ECC Agar
	Listeria		AOAC 2013.10
	Salmonella		AOAC 2013.01

(b) (6), (b) (4)

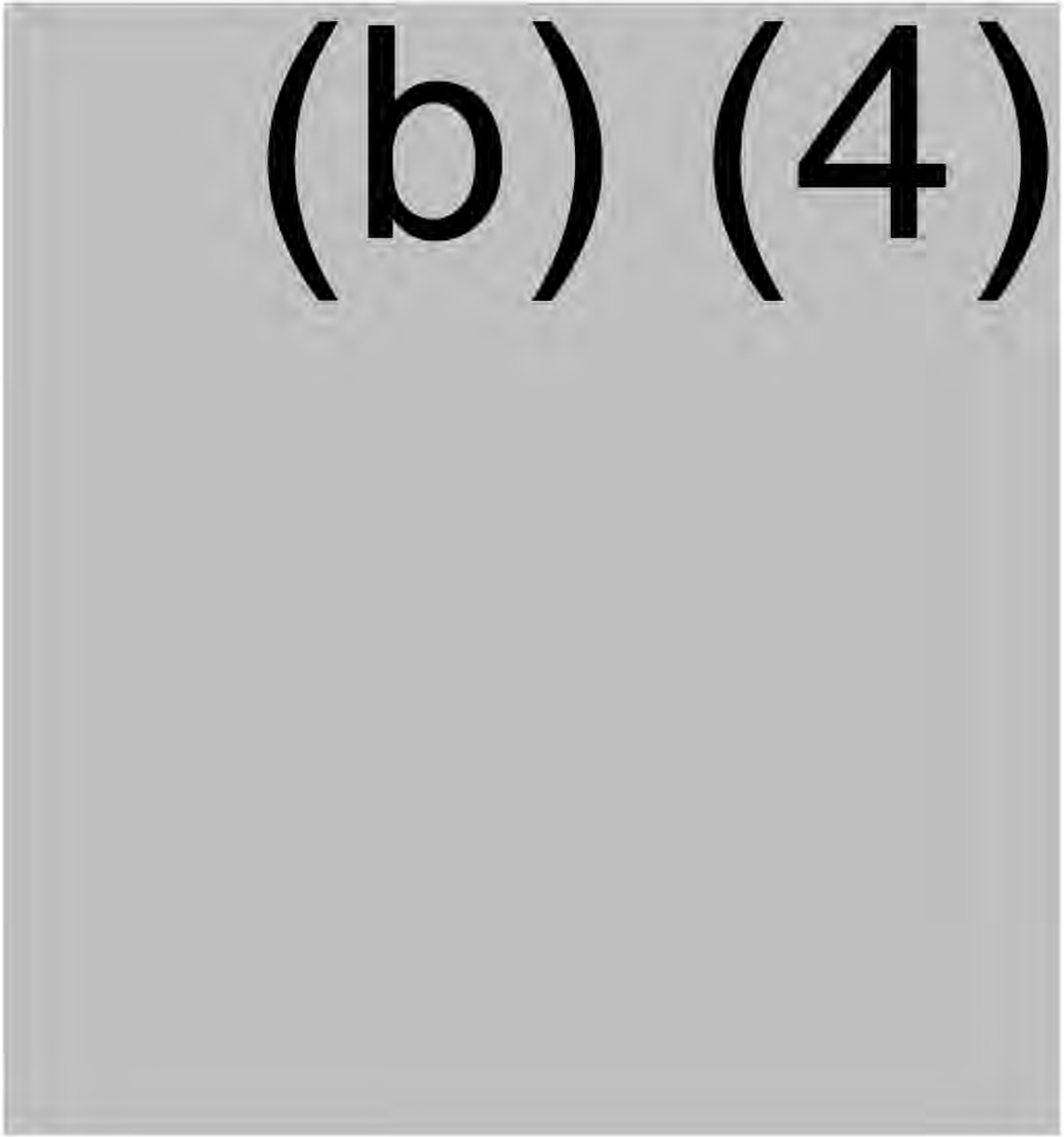
(b) (6), (b) (4)

Appendix 016Y: Scale-up of *Ruminococcus bovis* ASCUSDY10 from (b) (4) L to (b) (4) L CONFIDENTIAL

(b) (4)

Table 016Y.1: Research/Pilot Scale Batch Sizes at Each Stage of Manufacture (CONFIDENTIAL)

(b) (4)


(b) (4)

Appendix 017: Literature Search Strategy for *Ruminococcus bovis* ASCUSDY10

A literature search was conducted by Native Microbials on July 11, 2023 in order to identify potential information related to the safety and utility of *Ruminococcus bovis* as a direct fed microbial (DFM) strain for cattle. The overall search strategy is described in Table 1. The Google Scholar database was searched using the keyword/search terms listed in Table 2. The search was verified by reviewing the primary hits from a Google Scholar search.

Considering the number of articles identified (>500), the search results were reviewed to identify articles representative of the body of available data relating to the safety of the genus. In particular, the review focused on identifying comprehensive reviews, widely cited articles and recent articles of relevance.

Nomenclature

Ruminococcus bovis ASCUSDY10 is a novel species (Gaffney *et al.*, 2021). Because the lack of characteriziation bhy the scientific community under the new species name (*Ruminococcus bovis*), we performed a literature search based on the genus *Ruminococcus*. The relevant database was searched using the keyword/search terms listed in Table 2. The objective of the search was to identify a representative body of information on the genus *Ruminococcus*. It is important to mention that the search results include manuscripts mentioning both *Ruminococcus* Group I (including *R. bovis*) and Group II (different family and the scientific community has only been recently renamed to *Mediterraneibacter*) (Togo et al., 2018; Oren et al., 2019).

Table 1:	**Literature Search and Selection Strategy**	
Step 1	Records identified using selected literature databases	Google Scholar
	Total records (titles/abstracts) identified through electronic search (exclude patents and citations)	
Step 2	Exclude duplicates by searching Google Scholar using conditional terms	
Step 3	Screen titles/abstracts and exclude obviously irrelevant records	
Step 4	Review full texts and assess for relevance and eligibility for inclusion	

Table 2A: Topic Specific Search Terms using Genus [Ruminococcus]				
	Google Scholar Search		Google Scholar Search (exclude duplicates)	
	Input terms in search box	Results	Input terms in search box	Results
Search strategy for safety of species [Safety Search]	"Ruminococcus"+"tox"	413	"Ruminococcus"+"tox" OR "toxin" OR "toxins" OR "toxicity" OR "toxicities" OR "pathogen" OR "pathogens" OR "safe" OR "safety" OR "infection" OR "infections" OR "disease" OR "diseases" OR "mortality" OR "mortalities"	42,900
	"Ruminococcus"+"toxin"	6,570		
	"Ruminococcus"+"toxins"	7,410		
	"Ruminococcus"+"toxicity"	9,940		
	"Ruminococcus"+"toxicities"	748		
	"Ruminococcus"+"pathogen"	15,600		
	"Ruminococcus"+"pathogens"	19,500		
	"Ruminococcus"+"safe"	8,020		
	"Ruminococcus"+"safety"	11,400		
	"Ruminococcus"+"infection"	22,700		
	"Ruminococcus"+"infections"	15,000		
	"Ruminococcus"+"disease"	35,200		
	"Ruminococcus"+"diseases"	31,400		
	"Ruminococcus"+"mortality"	11,100		
	"Ruminococcus"+"mortalities"	294		
Search strategy for safety Ruminococcus [Target Animal]	"Ruminococcus"+"cattle"	10,900	"Ruminococcus"+"cattle" OR "cow" OR "cows" OR "bovine" OR "bovines" OR "ruminant" OR "ruminants" OR "calves" OR "calf"	21,500
	"Ruminococcus"+"cow"	7950		
	"Ruminococcus"+"cows"	7,910		
	"Ruminococcus"+"bovine"	12,000		
	"Ruminococcus"+"bovines"	418		
	"Ruminococcus"+"ruminant"	8,470		
	"Ruminococcus"+"ruminants"	9,210		
	"Ruminococcus"+"calves"	3,820		
	"Ruminococcus"+"calf"	2,060		
Search strategy for history of use of Ruminococcus	"Ruminococcus"+"food"	37,400	"Ruminococcus"+"food" OR "foods" OR "feed" OR "feeds"	38,400
	"Ruminococcus"+"foods"	15,200		
	"Ruminococcus"+"feed"	19,600		
	"Ruminococcus"+"feeds"	6,620		

Search: Must include quotes. Quotes ensure words being included in the search, although the words are not guaranteed to be in the found results. Exclude patents and citations.

References

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Microbiome Safety for *Ruminococcus bovis* ASCUSDY10

Objectives

The objective of this review is to:

a) Demonstrate that the typical microbial composition and diversity of the rumen microbial community of dairy cows is robust and stable across various diets and regions. We will demonstrate this by:

   i) Showing internal datasets (e.g. data and analyses created by Native Microbials)

   ii) Presenting data via external datasets (e.g. data published in peer reviewed manuscripts).

b) Present data that shows the feeding of native microorganisms does not negatively alter the microbiome composition. Specifically, that daily administration of *Ruminococcus bovis* ASCUSDY10 does not increase its own abundance nor the overall composition of the microbiome beyond typically observed ranges.

Robust Nature of the Dairy Rumen Microbiome

Native Microbials Rumen Microbiome Surveys: A series of experiments were conducted to obtain a representative sampling of the rumen microbiome composition. These samples were used to determine the typical ranges of abundances of rumen microorganisms under normal, farm-like conditions.

Survey 1: The first survey experiment identified the rumen composition of 8 mid-lactation Holstein dairy cows and 8 mid-lactation Jersey dairy cows over 28 days. The animals received both a typical (b) (6) farm diet and a diet that induces milk fat depression (see Attachment 1). Rumen samples were taken periodically throughout the study and analyzed for microbiome DNA and characterized accordingly.

Survey 2: The second survey experiment identified the rumen composition of 15 mid-lactation Holstein dairy cows over 28 days. The survey took place in (b) (6), and utilized a typical local diet as well as a milk fat depression inducing diet (see Attachment 2). Rumen samples were taken periodically throughout the study and analyzed for microbiome DNA and characterized accordingly.

Survey 3: The third survey experiment identified the rumen composition of 8 lactating Holstein dairy cows over 3x 19 days. The survey took place at (b) (6), (b) (4), and utilized diets with different forage to concentrate ratio of typical local ingredients (see Attachment 3). Rumen samples were taken periodically throughout the study and analyzed for microbiome DNA and characterized accordingly.

Findings: The results of both survey experiments are summarized together in Table 1, showing the average rumen bacterial phyla abundances. In all of these experiments, the abundances of the most predominant phyla were comparable to the ranges observed in the independent literature studies (presented below). The typical abundance of *R. bovis*, specifically, in the rumen of a dairy cow based on Native Microbials conducted rumen microbiome surveys was found to be ~0.0023%-28% of the rumen bacterial population.

Table 1. The abundance of major rumen bacterial phyla in the rumen from Native Microbials' microbiome survey 1, 2 & 3, reported as percent ranges. *Ruminococcus bovis* (98.5% sequence identity to *R. bovis* ASCUSDY10 16S rRNA gene) was detected in all animals.

	Abundance (%)		
	Survey 1	**Survey 2**	**Survey 3**
Phylum			
Actinobacteria	0.1-26	0.015-21	0.04-1.9
Bacteroidetes	4.6-77	13-73	2.3-55
Fibrobacteres	0.0067-15	0.0051-5.2	0.0078-11
Firmicutes	18-69	16-67	30-92
Proteobacteria	0.16-73	0.87-39	0.64-9.1
Spirochaetes	0.0098-25	0.017-4.9	0.0079-3.6
Tenericutes	0.018-3.8	0.006-2.7	0.0026-0.24
R. bovis*	0.0013-13	0.001 -13	0.082-10

Native Microbial Conducted Product Study: Native Microbials have also conducted a series of studies where native rumen microorganisms were administered daily in feed to dairy cows.

Study 1 (Valldecabres et al., 2022): 90 multiparous (2 or 3 lactation cycles) lactating Holstein cows (20-40 days in milk) were sourced from a large commercial dairy farm and housed in a single pen equipped with (b) (4) gates at (b) (6), (b) (4). The cows were divided into 3 groups, 30 of which was served as control (no microbes), 30 received a DFM consists of 2 microbes in feed daily (Group 1: no *R. bovis* ASCUSDY10), and the remaining 30 cows received a DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10). Both DFMs were in powder form and were homogeneously mixed into the feed prior to administration. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to lactating dairy cows daily for 39 weeks (Appendix 019A).

Study 2 (Goldsmith et al., 2023): 90 primiparous and multiparous lactating Holstein cows 92±23 days in milk were housed at (b) (6), (b) (4). The animals were divided into 3 groups, 30 of which were served as control (Control: no microbes), 30 received a DFM consists of 4 microbes in feed daily (Group 1: including *R. bovis* ASCUSDY10), the remaining 30 cows received another DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10 as well). Both DFMs were in powder form and were top-dressed onto the feed prior to

administration. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to lactating dairy cows daily for 112 days (Appendix 019B).

Study 3 (Dickerson et al., 2022): The third study was conducted at (b) (6), (b) (4) using 72 (1 additional cow as enrolled as backup) lactating primiparous and multiparous Holstein cows. The animals were divided into 3 groups, 24 of which were served as control (Control: received no microbes), 24 of which received a DFM consists of 2 microbes in feed daily (Group 1: no *R. bovis* ASCUSDY10), and the remaining 24 cows received a DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10). Both DFMs were in powder form and were top-dressed onto the feed prior to administration. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to lactating dairy cows for 20 weeks (Appendix 019C).

Study 4 (presented at conference: Marinho et al., 2022): This study was conducted at the (b) (4) using 117 lactating Holstein dairy cows. The animals were divided into 3 groups, 39 of which were served as control (Control: received no microbes), 39 of which received a DFM consists of 2 microbes in feed daily (Group 1: no *R. bovis* ASCUSDY10), and the remaining 39 cows received a DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10). Both DFMs were in powder form and were top-dressed onto the feed prior to administration. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to lactating dairy cows for 20 weeks (Appendix 019D).

Study 5 (presented at conference: Bulnes et al., 2022): This study was conducted at the (b) (4) using 60 Holstein dairy cows. The animals were divided into 2 groups, 30 of which were served as control (Control: no microbes), and the remaining 30 cows received a DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10). The DFM was in power form and was top-dressed onto the feed prior to administration. Native rumen microbes, including *R. bovis* ASCUSDY10 were administered to cows daily during the dry period (21±3 days precalving) and until 120±3 days in milk (Appendix 019E).

Study 6 (presented at conference: Ferro et al., 2022): This study was conducted at (b) (4) using 150 primiparous and multiparous Holstein cows. The animals were divided into two groups, 74 of which were served as control (Control: received no microbes), 76 of which received a DFM consists of 4 microbes in feed daily (Group 2: including *R. bovis* ASCUSDY10). The DFM was in powder form and was homogeneously mixed into the feed prior to administration. Native rumen microbes, *R. bovis* ASCUSDY10 was administered to lactating dairy cows for 20 weeks (Appendix 019F).

Findings: In these in-feed studies, it can be seen that the addition of *R. bovis* ASCUSDY10 to dairy cows daily for an extended period of time did not significantly alter the rumen bacteria composition when compared to the control group (Table 2). Abundances of major bacterial phyla were within standard ranges observed in animals not fed native rumen microbes. The average abundance of each major phylum tended to be similar across experimental groups.

	Abundance (%)																	
	Study 1			Study 2			Study 3			Study 4			Study 5		Study 6	
	Control	Group 1	Group 2	Control	Group 1	Group 2	Control	Group 1	Group 2	Control	Group 1	Group 2	Control	Group 1	Control	Group 1
***R. bovis* ASCUDY10 Administered?**	No	No	Yes	No	Yes	Yes	No	No	Yes	No	No	Yes	No	Yes	No	Yes
Phylum																
Actinobacterio	0.24 - 0.92	0.28 - 1	0.33 - 1	0.12 - 0.38	0.14 - 0.49	0.13 - 0.52	0.063 - 1.1	0.098 - 1.1	0.1 - 1.6	0.097 - 1.1	0.084 - 1.2	0.068 - 0.75	0.03 - 1.2	0.016 - 3.7	0.087 - 0.84	0.074 - 0.6
Bacteroidetes	34 - 53	28 - 58	25 - 53	44 - 71	36 - 64	37 - 69	23 - 77	20 - 78	24 - 80	33 - 84	36 - 85	36 - 82	33 - 81	25 - 84	41 - 70	33 - 70
Fibrobacteres	0.01 - 0.84	0.0054 - 0.69	0.013 - 0.63	0.0021 - 0.95	0.0021 - 1.7	0.0038 - 1.8	0.00088 - 0.98	0.0011 - 2.1	0.0013 - 2.1	0.0024 - 2.3	0.0024 - 0.74	0.0026 - 0.75	0.002 - 1.7	0.0043 - 2	0.0035 - 3	0.0041 - 4.3
Firmicutes	38 - 57	36 - 64	32 - 60	14 - 36	17 - 44	14 - 53	11 - 47	9.5 - 73	13 - 57	8.3 - 48	8.6 - 56	5.4 - 50	4.2 - 55	4.3 - 59	11 - 44	15 - 54
Proteobacteria	1.5 - 7.8	1.9 - 9.5	0.97 - 13	4.8 - 26	5.4 - 37	4 - 24	1.1 - 32	0.64 - 41	1.2 - 50	0.99 - 40	2.2 - 40	2.8 - 35	2.6 - 51	2.4 - 36	4.7 - 26	4 - 25
Spirochaetes	0.22 - 1.5	0.28 - 1.4	0.25 - 1.1	0.17 - 1.5	0.06 - 2.1	0.18 - 1.5	0.067 - 2.9	0.15 - 3.3	0.1 - 2.4	0.037 - 2.7	0.079 - 3	0.052 - 2.5	0.084 - 1.8	0.048 - 2	0.27 - 2.1	0.32 - 2.5
Tenericutes	0.29 - 0.91	0.28 - 1.1	0.17 - 1.4	0.18 - 1	0.085 - 1.5	0.26 - 1.8	0.078 - 4.2	0.075 - 13	0.011 - 4.3	0.13 - 2.3	0.15 - 3.3	0.12 - 1.8	0.11 - 3.3	0.1 - 2.9	0.19 - 2.7	0.32 - 3.6
R. bovis	4.7 - 12	2.9 - 12	3 - 12	1.6 - 11	1.8 - 9.6	1.4 - 13	0.41 - 6.1	0.16 - 13	0.09 - 11	0.15 - 15	0.4 - 14	0.37 - 16	0.014 - 16	0.007 - 16	0.51 - 14	0.34 - 7.1

Table 2. The percent abundance of major rumen bacterial phyla and *R. bovis* in the rumen from Native Microbials sponsored studies, reported as ranges of percent. *R. bovis* was detected in all animals, with and without in-feed *R. bovis* ASCUSDY10 administration.

Animal Experiments from Peer-Reviewed Literature: Peer reviewed manuscripts describing the bacterial rumen community using high-throughput, comprehensive bacterial community analyses were collected for further comparative analysis to establish the composition of the typical rumen and prevalence of *R. bovis.*

The following studies conducted by academic institutions reported the overall rumen microbiome composition of dairy and beef cattle: Jewell, et al. 2015, AlZahal, et al. 2017, Noel et al. 2017, Ribeiro et al. 2017, Petri et al. 2013. These manuscripts were selected based on the microbial marker selected for microbiome analysis (e.g. to maintain compatibility and consistency to internal analyses) and the breadth of diets represented in the analyses.

a) Jewell, et al. studied fourteen Holstein dairy cows across two lactation cycles. The major TMR components were corn silage, alfalfa haylage, high-moisture corn, dry corn, and roasted soybeans.

b) AlZahal, et al. investigated the role of dietary yeast on the rumen microbial community of 16 multiparous, lactating Holstein cows. The microbiome was characterized while the animals were fed both a high-forage and high-grain diet. The rumen solids, rumen fluids, and epimural microbial communities were analyzed.

c) Noel, et al. monitored the rumen microbiome of dairy cows grazing a rye-grass and clover pasture over 5 years.

d) Ribeiro, et al. transferred the rumen content of bison to 16 Angus x Hereford heifers to determine if the rumen microbiome could be altered. Heifers were fed a barley straw diet consisting of 70:30 forage-to-concentrate. Although both pre- and post-rumen transfer microbiome composition are reported in the manuscript, only the pre-transfer results are presented here.

e) Petri, et al. studied the rumen microbiome of 8 Angus heifers undergoing an acidosis challenge. Animals were fed a forage diet, a mixed forage diet, a high grain diet, a challenge diet, and a recovery diet. The microbiome was profiled for each diet.

f) McCann et al., 2016, McCabe et al., 2015, Meale et al. 2016, and Martinez-Fernandez et al. 2016 were also utilized to determine the abundance of *R. bovis* in cattle. Although their microbiome analyses were not robust enough to include in the analysis here, the raw reads used for their analyses were publicly available and thus could be used in internal analysis.

Findings:

i) The rumen microbial community composition is constantly in flux. The microbial population has been shown to change over time in response to a variety of factors, including diet composition, time after feeding, season, and stage of lactation. Additionally, there are groups of microorganisms that are unique to particular breeds of cow, regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Despite this variability, there is a core microbiome that appears in majority of animals. This core has been investigated at Native Microbials, as well as in independent academic studies. Although the results are variable at times, there are several phyla that tend to appear across all dairy cows (see Table 3 and Table 4).

Table 3. Abundance of major bacterial phyla in the rumen from independent studies, reported as a percent. Empty cells indicate that data was not reported for the phylum.

	Jewell (TMR)	Noel (Pasture)	Ribeiro (Barley straw)	Petri (Rumen Core*)	Petri (Forage)	Petri (High grain)	Petri (Acidotic)	Petri (Recovery)
Phylum								
Actinobacteria			1.78			1.6		
Bacteroidetes	49.42	11.8	20.29	32.8	25.7	40.3	40	31.5
Fibrobacteres		2.4	25.04		7.1			
Firmicutes	39.32	82.1	40.53	43.2	55.2	37	33.6	43.7
Lentisphaerae			1.35					
Proteobacteria	5.67		1.64	14.3	4.7	17.9	16.5	15.2
Spirachaetes			6.13		2.8			
Tenericutes	2.17							
Unclassified		1.5						
Other (low abundance)		2.2 (16 phyla)	0.08					

* Rumen core values reported in Petri, et al 2013 were sourced from Jouany 1991

Table 4. Abundance of bacterial phyla in the rumen of control animals from AlZahal, et al. 2017, reported as a percent.

Diet	**High Forage**			**High Grain**		
Rumen Sampling Location	**Solids**	**Fluid**	**Epimural**	**Solids**	**Fluid**	**Epimural**
Phylum						
Bacteroidetes	29.3	38	30	44.2	50.5	39
Firmicutes	15.4	13.5	21.9	27.3	23.3	22
Unclassified	18.8	15.8	23.6	13.1	11.6	17
Fibrobacteres	19	12.3	5.4	7.6	4.1	1.1
Proteobacteria	2.1	4.8	7.2	1.1	2.4	12.7
Tenericutes	6.2	3.9	3.5	1	0.8	0.7
Cyanobacteria	1.8	4.1	1.5	1.4	3	1.3
SR1	1.8	2	1.4	0.2	0.8	1.3
Spirochaetes	2.5	2	1.4	1.5	0.7	1

ii) The rumen microbiome is very plastic and highly responsive to external variables. Because of this, defining a normal healthy rumen is challenging. High-throughput bacterial community analyses were found for cattle and dairy cows fed a variety of diets (Jewell, et al. 2015, AlZahal, et al. 2017, Noel et al. 2017, Ribeiro et al. 2017, Petri et al. 2013). These manuscripts were further investigated to determine prevalence of the overall bacterial taxonomic composition of the

typical rumen microbiome. These studies showed that diet formulation has the greatest impact on microbiome composition.

iii) Cumulatively, these independent studies investigated the microbial community across a variety of breeds, diets, and feed management regimes. Lactating and non-lactating animals are also both represented. Table 3 (above) summarizes the findings from Jewell, et al. 2015, Noel et al. 2017, Ribeiro et al. 2017, and Petri et al. 2013 at the phylum level. Overall, Bacteroidetes and Firmicutes tended to dominate the rumen bacterial community, with the exception of the Ribeiro study in which *Fibrobacteres* also represented a substantial portion of the community. As can be seen from this data, there is a broad range of abundances. *R. bovis* ASCUSDY10 falls into the Firmicutes phylum, which was found to comprise 33-82% of the rumen microbial community.

iv) Despite the high variability in abundance, there does seem to be a typical range for the most predominant phyla. Overall, the observed abundance of Bacteroides within this group of healthy animals ranged from 11.8%-49.49%, while the observed abundance of Firmicutes ranged from 33.6%-82.1%. Other phyla did appear, but often represented less than 10% of the total bacterial population. These ranges were utilized to describe the average rumen in subsequent analyses.

While the above mentioned studies reported the overall rumen microbiome composition, the abundance of *R. bovis* could not be accurately determined due to either the sequence data was not available or the sequences were generated using non-Illumina platform (e.g., 454 or Ion Torrent has a greater error rate and lower coverage). Therefore, a separate list of published literature was selected based on: 1) the availability of Illumina generated sequences, 2) the variety of ruminants, and 3) the wide range of geographic locations. The abundance and prevalence of *R. bovis* are shown in Table 5.

Findings:

i) *R. bovis* was detected in all 19 studies conducted by the scientific community across the globe in 12 different countries. The abundance of *R. bovis* ranged from 1.9E-05 to 35%.

ii) *R. bovis* was detected in dairy and beef breeds, as well as sheep and buffalos, receiving diets containing various amount of concentrates.

iii) *R. bovis* was detected in nearly all sequence files (14,616 out of 14,637; 99.9%), suggesting it's naturally prevalent in ruminants (data associating sequence files to animals are not available).

iv) Out of all studies, it is important to mention that:

Table 5. The abundance and prevalence of *R. bovis* in published studies, reported in percent.

Ruminants	Diet	Location	Number of animals	*R. bovis* abundance (%)*	Number of sequence files	*R. bovis* prevalence (%)**	References
Beef feedlot cattle	0-50% concentrate	Australia	32	1.8-17	136	100	Martinez-Fernandez et al., 2016; Martinez-Fernandez et al., 2017; Martinez-Fernandez et al., 2018; Martinez-Fernandez et al., 2019
Beef feedlot cattle	50-100% concentrate	USA	32	0.1	1	100	Myer et al., 2016
Dairy cattle	0-50% concentrate	Austria, Germany, UK, Italy, Finland, Sweden	1028	0.013-21	2131	98.9-100.0	Wetzels et al., 2018; Schaeren et al., 2017; Wallace et al., 2019
Dairy cattle	0-50% concentrate (with linseed oil)	Netherlands	4	0-1.9E-05	3	33.3	van Lingen et al., 2017
Dairy cattle	0-100% concentrate	Austria	8	0.88-32	72	100	Neubauer et al., 2018
Dairy cattle	0-60% concentrate	Austria	8	6.5-35	32	100	Wetzels et al., 2016
Dairy cattle	50-100% concentrate	Austria, Germany, Spain, Italy	44	0.077-31	111	100	Wetzels et al., 2017; Deusch et al., 2017; López-García et al., 2018; Biscarinil et al., 2018
Dairy cattle	TMR	Denmark	750	0.00014-9.3	2318	99.8	Difford et al., 2018
Dairy cattle	Unknown	USA	Unknown	5.3-8.7	5	100	Nelson et al., 2014
Buffalo	Unknown	Italy	3	4.4-22	26	100	Chiariotti et al., 2018
Sheep	50-100% concentrate	New Zealand	22	0.38-4.3	45	100	Kamke et al., 2017

* The abundance of *R. bovis* is determined based on ≥98.5% 16S rRNA sequence similarity to *R. bovis* ASCUSDY10.

** The prevalence is determined based on the number of sequence files (from which *R. bovis* was detected) divided by the total number of sequence (SRA) files.

(1) Difford et al. (2018) studied the rumen microbiome of 750 commercial dairy cows. *R. bovis* was detected in 99.8% of the sequence files with an abundance ranged from 0.00014-9.3%.

(2) Wallace et al. (2019) studied the core rumen microbiome of 1016 dairy cows housed in different farms from UK, Italy, Finland, and Sweden. *R. bovis* was detected in 100% of the sequence files with an abundance ranged from 1.1-21.5%.

v) Therefore, *R. bovis* is naturally present in the rumen of ruminants consuming various diets across the globe, although its abundance varies.

Conclusion

This summary covers the Native Microbial studies as well as published data to assess the potential microorganisms shift in microbiome that may raise safety concerns. Information presented demonstrated that the normal microbial community in the rumen is robust and not adversely affected by the addition of *R. bovis* ASCUSDY10, which is a naturally occurring and prevalent rumen microorganism. Hence, it is clear that the dietary addition of *R. bovis* will not cause a safety concern based on changes in the microbiome**.**

Signed: _____ (b) (6) _____ Date: _____

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Attachment 1: Native Microbial's first survey experiment (Survey 1)

Diet: The survey took place in (b) (6), and utilized the following diet:

Ingredient	**g/100 g dry matter**
Alfalfa hay	7.79
Alfalfa green chop	5.98
Hay cubes	4.53
Corn silage	4.08
Wheat Silage	9.51
Almond Hulls	13.58
Citrus pulp	1.36
Wheat straw	0.89
Dry distiller's grains	10.41
Steamed rolled corn	22.54
Canola	5.41
Cottonseed	5.33
Millrun	5.88
Salt	0.46
Molasses + Mineral and vitamin mix	2.26
Chemical analysis	
Crude protein	17.26
Neutral detergent fiber	33.13
Acid detergent fiber	21.12

Animals were also induced into a milk fat depressed state by increasing the amount of concentrate in the diet. Although this report focuses on the microbial composition of healthy animals, this information has been included since independent research has also studied the bacterial composition of acidotic animals.

All animals were cannulated, and rumen samples were a composite sample comprised of rumen content collected from the dorsal, ventral, central, anterior, and posterior regions of the rumen. Samples were collected on Days 0, 1, 3, 6, 9, 10, 11, 14, 19, 22, and 28. Cows were observed daily for overall clinical health throughout the study.

Attachment 2: Native Microbial's second survey experiment (Survey 2)

Ingredient	**g/100 g dry matter**
Corn silage	37.0
Alfalfa haylage	17.3
Ground corn	9.2
Matrix corn	—
Roasted soybeans/SBM	5.2
Canola meal	9.4
Cookie meal	5.8
Grass hay/straw	5.4
Sugar cane molasses	2.3
Optigen / Urea	0.5
Cottonseed hulls	5.4
Mineral and vitamin mix	2.5
Chemical composition	**% DM**
CP	16.9
NDF	36.1
ADF	20.8
Starch	23.0

Animals were also induced into a milk fat depressed state by increasing the amount of concentrate in the diet. Although this report focuses on the microbial composition of healthy animals, this information has been included since independent research has also studied the bacterial composition of acidotic animal.

All animals were cannulated, and rumen samples were a composite sample comprised of rumen content collected from the dorsal, ventral, central, anterior, and posterior regions of the rumen. Samples were collected on Days 0, 3, 6, 9, 10, 16, 19, 22, and 28. Cows were observed daily for overall clinical health throughout the study.

Evaluating starch levels in sorghum based dairy heifer diets

(b) (6)
Animal Science Department
(b) (4)

Objectives 1: Evaluate starch levels and sorghum digestibility in dairy heifer diets.

Objectives 2: Evaluate the effect of F:C ratio with sorghum silage as a source of forage on utilization of protein in dairy heifers

Material and Methods

Eight Holstein heifers (12 months of age) will be fitted with a 10cm rumen cannula (Kehl, SP, Brasil) and will be used in a 3 x 4 Latin square design with 19 d periods including 15 d of adaptation and 4 d of sampling. The treatment will be 4 level of forage:concentrate (F:C) ratio (90:10, 80:20, 70:30, 60:40 respectively) using sorghum as the forage source. Each heifer will receive 3 of the 4 diets only. Heifers will be weighed weekly, and BW will be determined by the average of two measurements taken on the same day. The amount of TMR offered during the experiment will be adjusted on a weekly basis, based on BW. Heifers will be housed in individual tie-stall in a mechanically ventilated barn. Heifers will have free access to water and intake will be recorded during sampling days. The animals will be released for exercise 3h/d in paved pen (except sampling days). Intakes of feed, water and also health will be checked daily and recorded.

Diets

Rations will be designed to provide 0.22 Mcal ME/kg of $BW^{0.75}$ and 1.8 gN/kg of $BW^{0.75}$with the objective to obtain an ADG of approximately 1000g/d. Heifers will receive a diet in base of sorghum (sorghum silage SS) with different levels of F:C ratio. Dry matter of silages will be measured 3x/wk in a microwave as is described by (Pino and Heinrichs, 2014). The grain mixes will be formulated to provide the different levels of F:C and will be mixed before each period as

a single mix. Each diet will provide the same energy level and nitrogen intake in base of metabolic BW.

F:C Ratio	85-15	85-15	75-25	75-25	65-35	65-35	55-45	55-45
DMI, kg/d	7.48	7.44	6.78	6.67	6.95	7.01	6.76	6.40
MEI, Mcal/d	17.85	17.76	16.70	16.43	17.60	17.75	17.60	16.66
Mcal/kg	2.39	2.39	2.46	2.46	2.53	2.53	2.60	2.60
ME/kg BW0.75	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
CP%	13.91	13.91	13.80	13.80	14.20	14.20	14.59	14.59
RDP%	10.66	10.66	13.31	13.31	16.09	16.09	18.87	18.87
CPg/d:ME Mcal/d	56.08	56.08	56.02	56.02	56.05	56.05	56.04	56.04
NI	166.49	165.60	149.69	147.28	157.81	159.18	157.80	149.39
NI, gN/kg BW0.75	1.79	1.79	1.79	1.79	1.79	1.79	1.79	1.79
NDF%	44.96	44.96	41.05	41.05	37.12	37.12	33.18	33.18
% Starch	13.28	13.28	19.60	19.60	25.79	25.79	31.99	31.99
Starch I, gStarch/kg BW0.75	11.12	11.12	15.90	15.90	20.35	20.35	24.57	24.57
%BW	1.91	1.90	1.73	1.70	1.77	1.79	1.73	1.63

Ingredients: Sorghun silage, ground corn, canola meal, optigen and Mineral mix for all the diets.

Sample Collection and Analysis

Feedstuffs will be collected before every period and TMR daily to measure particle size during sampling days and will be composited by period and dried in an air force oven at 55°C for 48 h, and ground through a 1-mm screen ((b) (6), (b) (4)) for further analysis. The particle size of the diet will be analyzed daily ((b) (4) Particle Separator) before feeding and rate of consumption will be measured daily during the sampling days. Urine will be collected from d 15 to 20 using the collector described by (Lascano et al., 2010) only to avoid contamination of feces. Urine will be weighted daily after feeding and subsampled; composited urine samples will be acidified to pH < 2 by the addition of 12 M HCl and stored at -20 °C until analyses.

During sampling days, feces will be collected hourly and stored in airtight containers. After feeding, daily feces will be mixed and a subsample will be saved at 4°C and will be composited at the end of each period. Then, the subsample will he dried in an air force oven at 55°C for 72 h,

and ground through a 1-mm screen ((b) (6), (b) (4)) for further analysis.

The composited and dried feeds and fecal samples will be analyzed for DM, ash, CP and soluble CP (AOAC, 2000), NDF and ADF (Van Soest et al., 1991), and total C (Elemental analyzer). Analysis of NDF included use of heat-stable α-amylase (b) (6), (b) (4) and sodium sulfite (Van Soest etal., 1991) using an (b) (4)^{200} fiber analyzer (b) (4) (b) (6), (b) (4) Starch will be determined by the method of (Hall, 2008) previous reground of the samples to pass through a 0.5-mm screen. Soluble sugars will be measure by the same method. Rumen degradable protein will be estimated from calculation based on from ingredient values. Metabolizable energy intake will be estimated for each heifer within each period using the observed OM intake x 4.409 x 0.82 as described in (NRC, 2001). In addition, sorghum grain will be evaluated for prolamin (kafirin) concentration and will be compared with corn (zein).

In urine samples there will be determined: creatinine ((b) (4), uric acid (b) (4), allantoin (Chen, 1989), urea N (b) (4) and total N and C (Elemental analyzer). If ammonia interferes with urea N determination, it will be determined to correct the result of urea N (Chaney and Marbach, 1962). Urinary purine derivative (allantoin and uric acid) will be used for to estimate duodenal microbial N (Chen and Gomes, 1992).

Rumen fluid samples will be taken on days 18 from 5 locations in the rumen (dorsal, ventral, anterior, caudal, and central) at 0, 1.5, 3, 4.5, 6, 9, 13, 17, 21, 23 relative to feeding time. Two 15mL samples taken at each time point, one fixed and one not (see protocols).

Fixed cell protocol:

1. Prefill concial with 10% stop solution(95% Ethanol, 5% TRIzol/phenol)—i.e. for 15 mL conicals, prefill with 1.5 mL stop solution.

2. Fill with rumen sample to top of conical.

3. Mix conical by inverting several times.

4. Seal lid with parafilm, tape, etc. to ensure the sample doesn't spill during transit.

5. Store and ship at 4C.

Non-fixed cell protocol:

1. Fill conical to top with rumen sample.

2. Seal lid with parafilm, tape, etc. to ensure the sample doesn't spill during transit.

3. Store and ship at 4C.

We will prefill a concial with ~10-13mL PBS + 10% stop solution, and completely submerge a few mL of the fibrous material in this solution.

For the fixed samples: Sample all 8 cows, all time points.

For the non-fixed samples: Sample 2 cows (one 55:45, one 85:15), all time points.

Additional rumen fluid will be filtered through a 0.28-mm fiberglass mesh screen (b) (6) (b) (6), (b) (4). pH will be recorded (pH meter, model (b) (6), (b) (4) and strained fluid will be placed in 2 tubes; 1. 5 mL tubes with 1 mL 0.6% 2-ethylbutyric and 1 mL 25% metaphosphoric acid at -20°C for VFA analysis (Yang and Varga, 1989), 2. 10 mL tube with one drop of conc. HCl for ammonia determination (Chaney and Marbach, 1962) and total free AA content (Snell and Snell, 1954).

Fecal starch as an indicator of total-tract starch digestibility.

Recently, a study that determined total-tract starch digestibility (TTSD) using a single sample of feces was published. Near-infrared reflectance spectroscopy (NIRS) technique will be used to evaluate starch content in feces as described in (Fredin et al., 2014) considering NIRS-predicted FS%=0.4+(0.07 x FS%). Four fecal samples will be collected (rectal grab samples) at 6 h intervals starting at feeding time to evaluate the best sampling time. Samples will be weighed to not affect total fecal collections. Results will be compared in a linear regression with starch digestibility coming from feces total collection.

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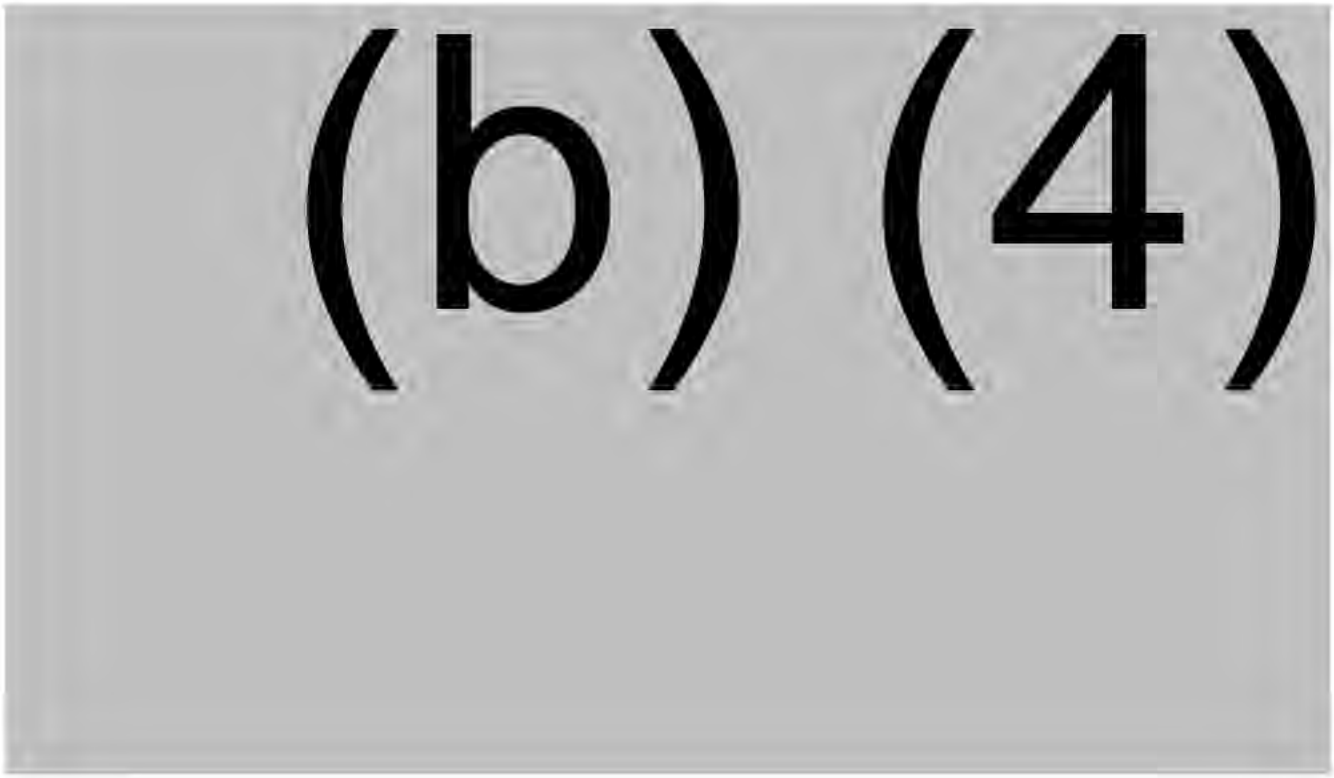
Animal Science (Agricultural Sciences) / (b) (4)
Evaluating Starch Levels in Sorghum Based Dairy Heifer Diets
Ascus Biosciences, Inc.
Project Dates: 05/15/2015 - 12/31/2015

	05/15/2015 - 12/31/2015	Total
Direct Costs		
(b) (4)		

Proposal: 26339
Generated by (b) (4) on: 05/17/2015

Budget Notes



Journal of Animal Science, 2022, 100, 1–10
https://doi.org/10.1093/jas/skac275
Advance access publication 30 August 2022
Ruminant Nutrition

Effects of rumen-native microbial feed supplementation on milk yield, composition, and feed efficiency in lactating dairy cows

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Abstract

The objective of this study was to evaluate the effects of two rumen-native microbial feed supplements (MFS) on milk production, milk composition, and feed efficiency. A total of 90 multiparous cows between 40 and 60 d in milk were enrolled in a randomized block design study. Within each block (baseline milk yield), cows were randomly assigned to: control (no microbial feed supplementation), MFS1 (0.33 g/kg total mixed ration [TMR] of an MFS containing a minimum of *Clostridium beijerinckii* at 2×10^6 CFU/g and *Pichia kudriavzevii* at 2×10^7 CFU/g), or MFS2 (0.33 g/kg TMR of a MFS containing a minimum of *C. beijerinckii* at 2×10^6 CFU/g, *P. kudriavzevii* at 2×10^7 CFU/g, *Ruminococcus bovis* at 2×10^7 CFU/g, and *Butyrivibrio fibrisolvens* at 2×10^7 CFU/g). Cows were housed in a single group and fed the study diets ad libitum for 270 d. Individual milk yield was recorded using electronic milk meters, and milk fat and protein were measured using optical in-line analyzers at each of two daily milkings. Treatment and treatment by time effects were assessed through multiple linear regression analyses. Treatment effects were observed for milk and energy-corrected milk (ECM) yields, milk fat and protein yields and concentrations, dry matter intake (DMI), and feed efficiency; those effects were conditional to time for milk yield, DMI, and feed efficiency. Overall, milk, ECM, fat, and protein yields were higher for MFS2 compared with control cows (+3.0, 3.7, 0.12, and 0.12 kg/d, respectively). Compared with MFS1, milk yield was higher and protein yield tended to be higher for MFS2 cows (+2.9 and 0.09 kg/d, respectively). In contrast, MFS1 cows produced 0.17 and 0.08 units of percentage per day more fat and protein than MFS2 cows, and 0.07 units of percentage per day more protein than control cows. Dry matter intake and feed efficiency were higher for MFS2 cows compared with MFS1 cows (+1.3 kg/d and 0.06, respectively), and feed efficiency was higher for MFS2 cows compared with control cows (+0.04). Where observed, treatment by time effects suggest that the effects of MFS2 were more evident as time progressed after supplementation was initiated. No effects of microbial supplementation were observed on body weight, body condition score, somatic cell count, or clinical mastitis case incidence. In conclusion, the supplementation of MFS2 effectively improved economically important outcomes such as milk yield, solids, and feed efficiency.

Lay Summary

This study evaluates the effects of two rumen-native microbial feed supplements (MFS) on milk yield, composition, and feed efficiency in lactating dairy cows. Ninety multiparous Holstein cows between 40 and 60 d in milk were assigned to control (no microbial feed supplementation), MFS1 (*Clostridium beijerinckii* and *Pichia kudriavzevii*), or MFS2 (*C. beijerinckii*, *P. kudriavzevii*, *Ruminococcus bovis*, and *Butyrivibrio fibrisolvens*) total mixed ration supplementation. Overall, MFS2 cows had higher milk and milk component yields than control and MFS1, while MFS1 cows had higher milk component concentrations than control and MFS2. Feed efficiency was higher for MFS2 compared with control and MFS1 cows. Microbial feed supplementation improved economically important outcomes such as milk yield, solids, and feed efficiency.

Key words: cattle, feed additive, microbial feed supplement

Abbreviations: BCS, body condition score; DIM, days in milk; DMI, dry matter intake; ECM, energy-corrected milk; MFS, microbial feed supplement; SCC, somatic cell count; TMR, total mixed ration

(b) (4)

Received March 27, 2022 Accepted August 30, 2022.

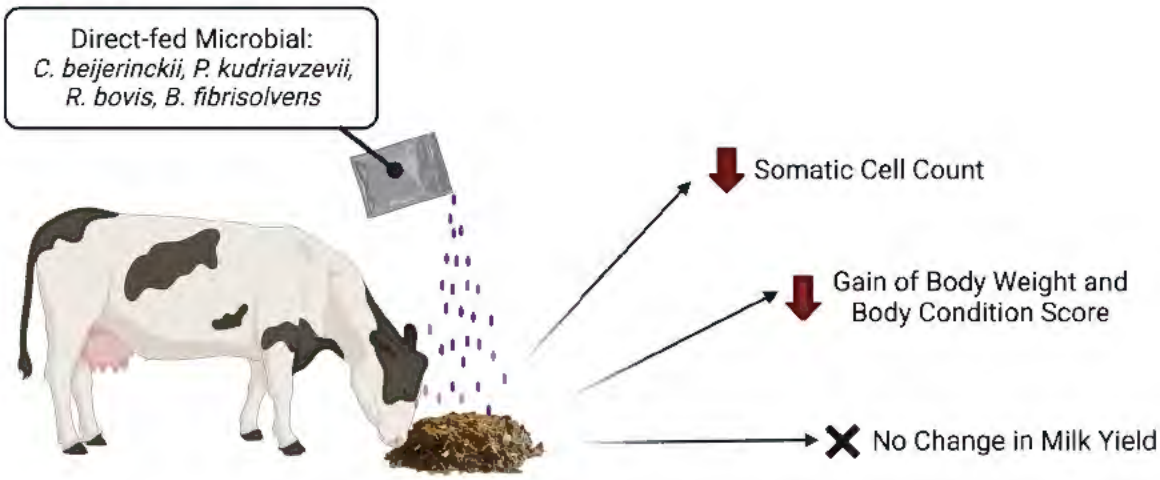
JDS Communications®
2023; 4

https://doi.org/10.3168/jdsc.2022-0250
Short Communication
Animal Nutrition and Farm Systems

The effect of supplementing native rumen microbes on milk production of dairy cows

Katelyn Goldsmith,[1] Josh Lefler,[2] Mallory Embree,[2] and Michael J. VandeHaar[1]*

Graphical Abstract



Summary

Direct-fed microbials (DFM) have been fed to dairy cows to improve milk production and efficiency. Direct-fed microbials commonly contain microorganisms that are not native to cows. We evaluated the effects of 2 DFM containing native rumen microorganisms on milk production of dairy cows. The supplements did not alter yield of total milk, protein, or fat, but decreased SCC and BW gain, and tended to decrease feed intake and increase energy-corrected milk/dry matter intake. Overall, native DFM treatment had little effect in this study.

Highlights

- Direct-fed microbial (DFM) supplementation had no effect on milk production.
- Body weight gain and BCS gain were lower in cows fed supplemental DFM.
- DFM supplementation did not alter digestibility of NDF, starch, or CP.
- DFM supplementation did not significantly alter plasma metabolite concentrations.
- DFM supplementation decreased SCC.

[1]Department of Animal Science, Michigan State University, East Lansing 48824, [2]Native Microbials Inc., San Diego, CA 92121. *Corresponding author: mikevh@msu.edu. Received May 03, 2022. Accepted August 26, 2022.

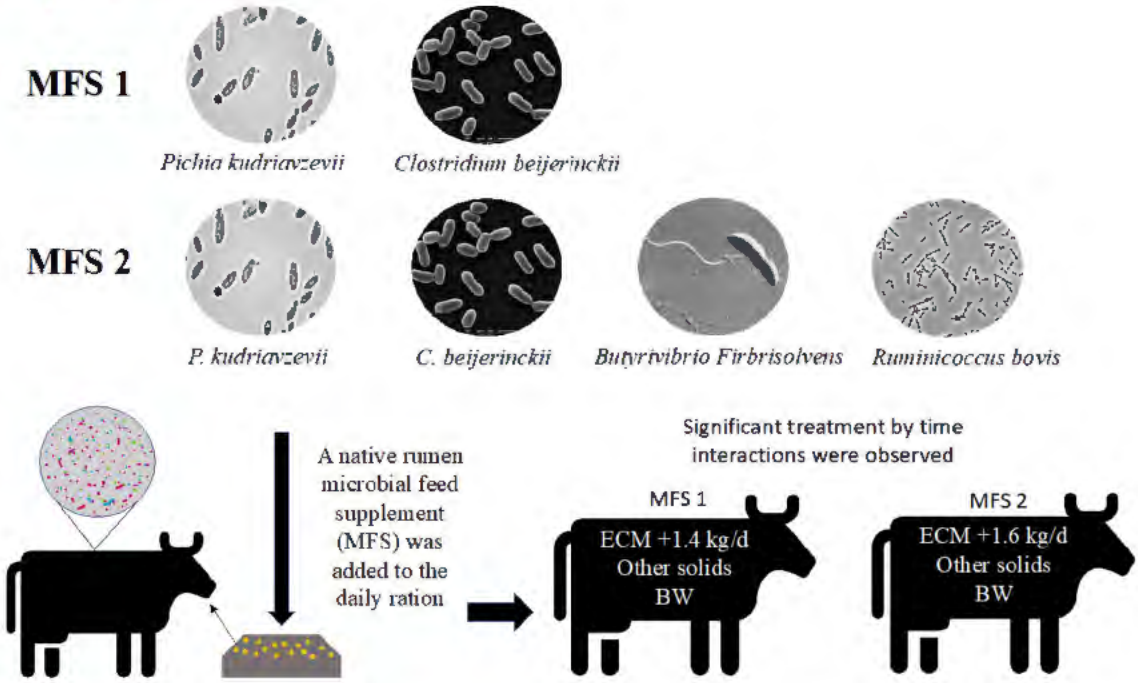
JDS Communications®
2022; 3:239–244

https://doi.org/10.3168/jdsc.2022-0210
Short Communication
Animal Nutrition and Farm Systems

Feeding native rumen microbial supplements increases energy-corrected milk production and feed efficiency by Holstein cows

A. M. Dickerson,[1] F. Yang,[2] H. B. Green,[2] M. M. Embree,[2] and J. K. Drackley[1]*

Graphical Abstract



Summary

This study tested the effect of 2 microbial feed supplements consisting of microorganisms sourced from the rumen on production of Holstein dairy cows. Supplementation improved energy-corrected milk production, and the response to the 4-microbe supplement was greater than the 2-microbe supplement. Greater production improvements occurred in cows that started receiving microbes earlier in lactation, especially in the group receiving the 4-microbe supplement.

Highlights

- Supplementation with native rumen organisms improves energy-corrected milk production
- The 4-microbe supplement performed better than the 2-microbe supplement
- No negative impact on health or body weight from supplementation
- Production improvements may be influenced by lactation stage

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Dietary supplementation of rumen native microbes improves lactation performance and feed efficiency in dairy cows

Journal:	*Journal of Dairy Science*
Manuscript ID	Draft
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Santos, José; University of Florida, Department of Animal Sciences Marinho, Mariana; University of Florida, Animal Sciences Perdomo, Milerky; University of Florida, Animal Sciences Simões, Bruna; University of Florida, Animal Sciences Husnain, Ali; University of Florida, Animal Sciences Arshad, Usman; University of Florida, Animal Sciences; University of Wisconsin-Madison, Animal and Dairy Sciences Figueiredo, Caio C.; Washington State University, Department of Veterinary Clinical Sciences
Key Words:	dairy cow, feed efficiency, microbial additive, production

Protocol – (b) (4)

Dry and Lactating Cow with Galaxis 2.0

(b) (4)

(b) (6), (b) (4)

Date 02/17/2021

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(b) (6)
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Mallory Embree
Sponsor Representative
Native Microbials

Date 2/17/2021

1 Project Description

This research will be conducted at the (b) (6), (b) (4) All cows will be housed in a naturally ventilated barn(s) with access to free-stalls bedded with straw.

2 Experimental Design

2.1 Animals and Treatments

Sixty Holstein dairy cows, will be assigned to one of two treatments (n = 30 cows/trt ; Control, Galaxis 2.0) starting from the dry (21 ± 3 days precalving) period until 140 ±3 days in milk. Animals should be evaluated for soundness and removed before beginning the trial if have feet or leg issues, are a three quartered animal, carrying twins or don't adapt to the (b) (4) gates during the training period. Animals will be removed from trial after calving if have one of the following conditions: Injury from calving that may affect production such as C-Section, leg paralysis, displaced abomasum, or other conditions affecting the animals to complete the trial.

2.1.1 Blocking

Treatments will be blocked and balanced for expected calving date, parity, previous 305 ME (or genetic merit for heifers).

Parity:

- Primiparous (no more 25% (14) of animals)
- Multiparous (2^{nd}, 3^{rd} or 4^{th})

Previous lactation milk production or predicted milk :

- Within a block should be a tight as possible

Minimum Milk production during first 21 days

- Cows and heifers may be removed from trial at 28± 3 DIM if production doesn't meet the following production levels.
  - Cows averaging < 45.4 lbs/day during 14 to 28 days in milk
  - Heifers averaging < 33.6 lbs/day during 14 to 28 days in milk
  - Animals will be replaced in the block if animals removed for production

Cows will be adapted to (b) (4) gates in dry barn and then baseline data (covariate) will be collected for 1 week prior to start treatments for all cows. Cows will remain on their respective treatment diets until 140 ± 3 days in milk. Cows will be housed in a bedded pack loose housing

during dry period and free-stall barn during lactation where they will be fed individually using the (b) (4) gate system with individual transponders. General cow housing and care comfort are in line with the current SOP at the (b) (4)

Treatments will consist of:

1. Control, (150 g of ground corn carrier) starting at 21± 3 days pre-calving
2. Galaxis 2.0, (5 g of Galaxis 2.0/cow/d plus 150 g of ground corn carrier) starting at 21± 3 days pre-calving

A top-dress for each cow will be produced daily by adding the 5 g treatment to approximately 150 g of a carrier such as ground corn. Treatment containers will have cow number and color code to ensure delivery to the correct cow. Treatments will be top-dressed on the feed and mixed into the top 2-6 inches of the TMR. Treatments will be color coded and marked on the stalls and containers delivering the top-dress to the cows. Personnel will change gloves between treatments.

Native Microbials will supply treatments in packets.

2.1.2 Treatments

2.1.2.1 Handling of Packets

- Treatments will be packed into daily packet, with each packet containing enough product for each day's feeding for each treatment plus 10% extra. Packets should be stored at 4°C at (b) (4).
- Use a fresh packet for each day and weigh out each cow's dose individually and mix into approximately 150 g of ground corn. Reseal the packet, label with the date opened, and store at 4°C.
- Approximately every 50-60 days one unused packet along with used packets will be sent to Native Microbials for assay. Ship open packets with unopened packet and should be shipped on Monday or Tuesday via overnight with ice packs (Native will provide payment method).
- Ship to (b) (6) 10255 Science Center Drive, Suite C2, San Diego CA 92121. Please send an email to (b) (6) with tracking number when shipping product on a Monday or Tuesday with next day shipping

2.1.3 Feeding

The basal diet will be formulated to meet or exceed dairy NRC nutrient requirements. The basal TMR will be delivered to barn and (b) (4) used to weigh out individual animal feed. Individual cow dry matter intake (DMI) will be adjusted daily to allow for a 10% feed refusal rate. The basal diet will not include any yeast culture or yeast-based additives.

The 5 g of test product will be mixed with ground corn to improve accuracy. The test products will be hand-fed once daily, top-dressed on each cow's individual TMR diet.

3 Observations

3.1 Dry Matter Intakes

Dry matter intakes from 21± 3 days prepartum to 140 ± 3 days in milk using (b) (4) system (daily/weekly). Diets will be offered at ad libitum intake with 10% refusals. Orts will be weighed daily by (b) (4) personnel and daily intakes calculated for the duration of the study. Cows should be fed at a reasonably consistent time each day (approximately 9 am), which will be determined by our group and the (b) (4) personnel. Each cow will have an individual (b) (4) feed tub to separate feed from other cows.

3.2 Body Weight(s)

Double body weights will be collected at beginning of study, Calving (within 24 hours) and 1 DIM (24 to 48 hours), 12± 3 DIM and every 28 days and at removal from trial. More frequent body weights are allowed and will be defined by each trial site but the double body weights are a requirement. Average body weight change will be calculated by 28-day periods and overall body weight change based on body weight at end of covariate.

3.3 Body Condition Score

(BCS)1-5 (b) (4) scoring system: Two scorers at beginning of study Calving and 1 DIM, 28 ± 3 DIM and every 28 days and at removal from trial. Average BCS will be determined and used for analysis. If BCS is ≥0.5 between scorers, then scorers will independently rescore animal.

3.4 Milk Composition

Once a week during lactation, a milk sample per animal will be collected at each milking during a 24-hour period. Milk samples will be collected on the same day(s) of the week. Milk samples will not be composited but will be sent to (b) (4) laboratory located at (b) (6) for analysis of milk fat, protein, lactose, total solids, MUN and somatic cell counts.

3.5 Milk Production

Daily milk weights will be collected at each milking by a parlor observer and compared to the milk weights captured by the (b) (4) milking system and milk weight reconciliation will occur daily. Cows are milked in a D-8 rapid exit parallel milking parlor. A milking system maintenance including calibration of the meters will be performed prior to start of trial. Average daily milk and ECM by week and total treatment period will be calculated based on milk and milk composition data collected on the trial.

3.6 Feed Efficiency

Weekly feed efficiency will be calculated as milk/DMI and ECM/DMI during lactation.

3.7 Feed Sampling

Weekly silage samples will be collected for particle separation and a NIR nutrient analysis. Dry matter determinations will be conducted on corn silage and wet forages twice a week. TMR may be adjusted based on these dry matter determinations. Concentrate mixes will be sampled and analyzed. TMR samples will be collected weekly, composited monthly and analyzed by NIR.

3.8 Health and Reproduction

All health (including mastitis) and reproductive events and treatments will be captured in (b) (4) system throughout the trial and summarized by treatment. The experiment will use the approved herd health plan for the (b) (4) Unit, as well as the standard operating procedures for on-farm treatments. The dairy health program was reviewed by the dairy health working group and approved by Institutional Animal Care and Use Program. All reproductive events will be captured in (b) (4) system.

3.9 Physiological and Metabolic Observations on Subset of Animals/Trt/Group (12 multiparious cows /Trt/Group)

1. Plasma heparin tubes (metabolites): Glucose, NEFA, BUN, and BHBA at twice a week before calving and three times a week after calving until 30 days in milk. Plasma health related biomarkers will be analyzed for inflammation, liver function, and oxidative stress at same time points.

2. Rumen pH, VFA, ammonia (NH3) analyses will perform weekly from -14 day to 21 DIM from each animal on all animals on study.

3. Rumen microbial population via RT-qPCR analysis of at least 17 rumen bacterial species will be performed weekly from -14 day to 21 DIM from each animal on the 12 or more animals on study.

4. Microbiome sampling: (12/animals/trt group): Animals will be sampled twice (-14 ± 3 and -7 ± 3 precalving) during dry and three times (7 ± 3, 14 ± 3 and 21 ± 3) post calvings, and approximately 70 days in milk and 100 days in milk for microbiome analysis

(b) (4)

(b) (4) (DC)

(b) (4)

Farm: Native Microbials
Cattle: DC
Barn/Lot: Dry Cows

FBW: 1570 lbs
BCS (1-5): 3.25
ADG: 0.000 lbs/day

Inputted DMI: 28.60 lbs
Predicted DMI: 31.02 lbs

Ration Fed					
Ingredient	$/hd	%DM	DM lbs/day	AF lbs/day	Price $/Ton
2 CORN SILAGE 2020	0.00	27.8	11.5	41.4	0.0
5 Grass hay Feb2021	0.00	94.1	6.2	6.6	0.0
4 Straw Feb2021	0.00	95.1	2.9	3.0	0.0
Soybean Meal 47.5 Solvent	0.00	90.0	0.000	0.000	0.0
Dry Cow Mix Native	0.00	91.2	7.9963	8.7649	0.0
Water	0.00	0.1	0.0000	0.0000	0.0
Totals	0.00	47.8	28.5963	59.7699	

Output	Value
CP (%)	15.00
RDP (%DM)	10.96
RUP (%DM)	4.04
MP Supply (g)	1209.79
LYS (%MP)	7.17
MET (%MP)	2.29
LYS:MET	3.13
LYS:ME	2.80
MET:ME	0.89
Forage (%DM)	72.04
ADF (%DM)	27.06
aNDFom (%DM)	41.77
peNDF (%DM)	33.46
NFC (%DM)	28.20
Starch (%DM)	15.14
Sugar (%DM)	3.47
Soluble Fiber (%DM)	6.31
Ferm. CHO (%DM)	46.45
Lignin (%DM)	2.85
EE (%DM)	3.57
ME Conc. (Mcal/lb)	1.04
Ca (%DM)	1.42
P (%DM)	0.31
Mg (%DM)	0.56
K (%DM)	1.23
S (%DM)	0.38
Na (%DM)	0.11
Cl (%DM)	0.73
Salt (%DM)	0.12
DCAD1 (meq/kg)	-84.23
Vit-A (KIU)	127.46
Vit-D (KIU)	42.52
Vit-E (IU)	1033.51
Monensin (mg/day)	0.00
Urea (lbs)	0.00
ME Allowable Gain (lbs/day)	0.00
MP Allowable Gain (lbs/day)	0.00
uNDF (%DM)	8.74

(b) (4)

Formulation Mix Report: Dry Cow Mix Native

Farm: Native Microbials **Thursday, February 11, 2021**

Ingredient Detail (Imperial)

		As-Fed Amount	Ingredient DM Percent	Dry Matter Amount	% of As-Fed	As-Fed (lbs/Ton)	$/Ton
02027	Soybean Meal 47.5 Solvent	3.97	90.00	3.57	45.31	906.27	0.00
02008	Corn Dist Ethanol	1.19	88.80	1.05	13.54	270.84	0.00
01103	Soybean Hulls Ground	1.01	91.00	0.92	11.57	231.44	0.00
05034	Limestone Ground	0.67	99.50	0.67	7.63	152.65	0.00
11024	Biochlor	0.62	87.00	0.54	7.04	140.75	0.00
01039	Corn Grain Ground Fine	0.44	88.00	0.38	4.97	99.40	0.00
05039	Magnesium Sulfate 7H2O (Epsom Salts)	0.17	99.50	0.17	1.91	38.17	0.00
11055	(b) (4) Choline	0.13	98.00	0.13	1.50	30.08	0.00
05009	Calcium Chloride Dihy	0.11	99.50	0.11	1.27	25.45	0.00
05038	Magnesium Ox	0.11	99.50	0.11	1.27	25.45	0.00
05086	Vitamin E	0.07	99.50	0.07	0.77	15.39	0.00
05016	Calcium Sulfate Dihyd	0.07	99.50	0.07	0.76	15.27	0.00
05053	(b) (4) Dairy Vitamin Premix	0.05	96.00	0.05	0.61	12.12	0.00
05014	Calcium Phosphate Mono (b) (4)	0.05	99.50	0.05	0.55	10.99	0.00
11057	Chromium 4 percent premix	0.05	95.00	0.04	0.54	10.70	0.00
05067	Salt White	0.03	99.50	0.03	0.38	7.65	0.00
05053	(b) (4) Dairy TM Premix	0.03	96.00	0.03	0.37	7.38	0.00
		8.76		**8.00**	**100.00**	**2000.00**	

(b) (4)

(b) (4) (LDC)

(b) (4)

Farm: (b) (4)
Cattle: LDC
Barn/Lot: Lactating Cows

FBW: 1513 lbs
BCS (1-5): 3.00
ADG: 0.117 lbs/day

DIM: 70
Milk: 84.9 lbs/day
Milk Fat: 3.70%
Milk Prt: 3.20% (True) / 3.44% (Crude)

Inputted DMI: 55.52 lbs
Predicted DMI: 52.20 lbs

Ration Fed					
Ingredient	$/hd	%DM	DM lbs/day	AF lbs/day	Price $/Ton
2 CORN SILAGE 2020	0.00	27.8	19.2	69.1	0.0
3 ALFALFA Feb2021	0.00	89.0	8.1	9.1	0.0
1 COTTON SEED Fed2021	0.00	90.7	4.000	4.410	0.0
QLF 60 38	0.00	60.3	2.600	4.311	0.0
Water	0.00	0.1	0.0000	0.0000	0.0
Smartamine M	0.00	98.0	0.000	0.000	0.0
4 Straw Feb2021	0.00	95.1	1.5	1.6	0.0
Native Lac Mix	0.00	89.8	20.1200	22.4157	0.0
Totals	0.00	50.1	55.5190	110.8794	

Output	Value
CP (%)	16.84
RDP (%DM)	10.18
RUP (%DM)	6.65
MP Supply (g)	2893.30
LYS (%MP)	6.65
MET (%MP)	2.12
LYS:MET	3.14
LYS:ME	2.93
MET:ME	0.93
Forage (%DM)	51.87
ADF (%DM)	20.03
aNDFom (%DM)	30.17
peNDF (%DM)	22.59
NFC (%DM)	40.95
Starch (%DM)	25.36
Sugar (%DM)	6.63
Soluble Fiber (%DM)	6.09
Ferm. CHO (%DM)	43.54
Lignin (%DM)	3.37
EE (%DM)	5.09
ME Conc. (Mcal/lb)	1.18
Ca (%DM)	0.87
P (%DM)	0.36
Mg (%DM)	0.37
K (%DM)	1.16
S (%DM)	0.24
Na (%DM)	0.49
Cl (%DM)	0.59
Salt (%DM)	0.41
DCAD1 (meq/kg)	197.80
Vit-A (KIU)	130.41
Vit-D (KIU)	43.51
Vit-E (IU)	613.30
Monensin (mg/day)	359.59
Urea (lbs)	0.18
ME Allowable Milk (lbs/day)	94.60
MP Allowable Milk (lbs/day)	93.98
uNDF (%DM)	5.46

(b) (4)

Formulation Mix Report: Native Lac Mix

Farm: (b) (4) **Thursday, April 01, 2021**

Ingredient Detail (Imperial)

		As-Fed Amount	Ingredient DM Percent	Dry Matter Amount	% of As-Fed	As-Fed (lbs/Ton)	$/Ton
01039	Corn Grain Ground Fine	5.87	88.00	5.16	50.81	1016.15	0.00
02027	Soybean Meal 47.5 Solvent	2.29	90.00	2.06	19.82	396.49	0.00
08029	Soy Best	1.63	89.00	1.45	14.10	282.06	0.00
02008	Distillers dry	0.51	88.80	0.45	4.41	88.10	0.00
05070	Sodium Bicarbonate	0.31	99.50	0.31	2.69	53.77	0.00
05034	Limestone Ground	0.28	99.50	0.28	2.42	48.46	0.00
09006	Energy Booster 100	0.20	99.36	0.20	1.76	35.26	0.00
05067	Salt White	0.11	99.50	0.11	0.93	18.50	0.00
02039	Urea 281 CP	0.09	99.00	0.09	0.79	15.85	0.00
01103	Soybean Hulls Ground	0.07	91.00	0.06	0.62	12.34	0.00
05038	Magnesium Ox	0.06	99.50	0.06	0.53	10.58	0.00
05014	Calcium Phosphate Mono (b) (4)	0.06	99.50	0.06	0.53	10.58	0.00
05053	(b) (4) Dairy TM Premix	0.03	96.00	0.03	0.24	4.85	0.00
05053	(b) (4) Dairy Vitamin Premix	0.03	96.00	0.03	0.24	4.85	0.00
05086	Vitamin E	0.01	99.50	0.01	0.09	1.77	0.00
11145	Biotin 2 per	0.00	99.00	0.00	0.02	0.39	0.00
		11.55		**10.36**	**100.00**	**2000.00**	

(b) (4)

Nutrient Analysis

	DM	As-Fed		DM	As-Fed		DM	As-Fed
Crude Protein (%)	25.45	22.84	Organic Co (ppm)	0.00	0.00	Choline Added (mg/lb)	0.00	0.00
Sol. CP (%CP)	24.58	22.06	Cu Added (ppm)	27.07	24.29	Dry Matter (%)	89.75	-
RUP (%CP)	33.82	30.36	Cu Total (ppm)	36.23	32.52	NFC (%DM)	47.27	42.42
RDP (%CP)	66.18	69.64	Organic Cu (ppm)	0.00	0.00	Sugar (A4) (%DM)	4.69	4.21
Fat Total (%DM)	6.05	5.43	I Added (ppm)	2.70	2.42	Starch (B1) (%DM)	38.00	34.10
Fat Veg Unpr (%DM)	1.93	1.73	I Total (ppm)	2.74	2.46	Sol. Fiber (B2) (%DM)	4.59	4.12
ADF (%DM)	5.13	4.60	Fe Added (ppm)	179.68	161.26	ADFIP (%DM)	2.73	2.45
aNDFom (%DM)	11.11	9.97	Fe Total (ppm)	263.51	236.49	NDFIP (%DM)	9.38	8.41
NEl (Mcal/lb)	0.77	0.69	Mn Added (ppm)	137.74	123.61	peNDF (%NDF)	26.53	23.81
NEg (Mcal/lb)	0.54	0.48	Mn Total (ppm)	154.92	139.03	peNDF (%DM)	2.95	2.64
NEm (Mcal/lb)	0.80	0.72	Organic Mn (ppm)	0.00	0.00	Lignin (%DM)	1.13	1.01
Ash (%DM)	11.74	10.53	Se Added (ppm)	0.83	0.75	Monensin (mg/lb)	0.00	0.00
Ca (%DM)	1.24	1.11	Se Total (ppm)	0.93	0.83	Chlortetracycline (mg/lb)	0.00	0.00
P (%DM)	0.55	0.49	Organic Se (ppm)	0.00	0.00	Decoquinate (mg/lb)	0.00	0.00
Salt (%DM)	1.03	0.92	Zn Added (ppm)	162.57	145.90	Lasalocid (mg/lb)	0.00	0.00
Na (%DM)	1.23	1.10	Zn Total (ppm)	201.82	181.12	MGA (mg/lb)	0.00	0.00
Cl (%DM)	0.67	0.61	Organic Zn (ppm)	0.00	0.00	Oxytetracycline (mg/lb)	0.00	0.00
Mg (%DM)	0.57	0.52	DCAD (Meq/kg)	449.09	403.04	Tylosin (mg/lb)	0.00	0.00
K (%DM)	1.05	0.95	Vit A Added (KIU/lb)	6.48	5.82	Biotin (mg/lb)	1.94	1.74
S (%DM)	0.27	0.24	Vit D Added (KIU/lb)	2.16	1.94	ME (Mcal/lb)	1.20	1.07
Co Added (ppm)	1.68	1.51	Vit E Added (IU/lb)	30.48	27.36			
Co Total (ppm)	1.72	1.55	Niacin Added (mg/lb)	0.00	0.00			

not increase during P2, but lipopolysaccharide binding protein (LBP) progressively increased during HS and was increased (60%; $P < 0.01$) on P2d5 compared with P1. LBP remained elevated during P3 compared with P2 and SAA increased (61%; $P < 0.01$) during P3 and neither were affected by Zn source. In P3, DMI rapidly increased compared with HS, but this increase tended to be more pronounced (10%; $P = 0.06$) in HYD compared with CON. HS induced GIT hyperpermeability and this was associated with an inflammatory response. Circulating Cr differences during acute HS implies that Zn-HYD may specifically benefit the proximal sections of the GIT.

Key Words: leaky gut, Cr-EDTA

1437 Effects of heat stress on inflammation and intestinal integrity in dairy calves. Z. Yu*, J. M. Cantet, and A. G. Ríus, *Department of Animal Science, University of Tennessee Institute of Agriculture, Knoxville, TN.*

Heat exposure can increase intestinal permeability and induce local and systemic inflammatory pathways in mammals. Therefore, the objective of this study was to evaluate how prolonged heat stress affects the integrity of intestinal epithelium and the expression of inflammatory response-related components in Holstein bull calves. Twelve week-old calves were individually housed in temperature-controlled rooms and assigned to 1) heat stress conditions and fed ad libitum (HS, ~36.0°C of ambient temperature for ~10 h/d, 26 to 45% relative humidity, n = 8) and 2) thermoneutral conditions and restricted starter intake (TN, constant ambient temperature of 19.5°C, 28 to 46% relative humidity, n = 8) for 7 d. Blood samples were collected to measure concentrations of plasma cytokines to assess the tone of systemic inflammation. Calves were euthanized and samples of jejunum, ileum and colon were harvested and flash-frozen to subsequently evaluate gene and protein expressions (RT-qPCR and automated Western Blots), activity of myeloperoxidase (MPO), and cytokine concentrations (Multiplex immunoassays). Plasma cytokine analysis were conducted using conventional ELISA. Data were analyzed using the PROC MIXED procedure in SAS with treatment as the fixed effect. Relative to TN, HS increased the concentration of interleukin 36 receptor antagonist by 3.5-fold ($P < 0.05$). Conversely, HS decreased concentrations of IL-1α in jejunum and IL-6 in plasma (36% and 33%, respectively; $P < 0.05$) The expression of TJP1 decreased 70% in jejunum of HS calves ($P < 0.05$); however, the expression of HP2 tended ($P = 0.098$) to increase in HS calves. The expression of HSF-1 which plays a key role in the regulation of heat shock response was decreased in jejunum of heat-stressed calves (48.08%; $P < 0.05$). The activity of MPO was not affected. Our results suggest that 7 d of heat stress elicited an anti-inflammatory response which may alleviate some of the negative effects of heat stress in dairy calves.

Key Words: heat stress, tight junction, inflammation

1438 Effects of supplementing native rumen microbes on milk production of mid-lactation dairy cows. K. Goldsmith*[1], J. Liesman[1], J. Lefler[2], and M. VandeHaar[1], [1]*Michigan State University, East Lansing, MI,* [2]*Native Microbials, Inc., San Diego, CA.*

Our objective was to evaluate the effects of a direct-fed microbial (DFM) supplement containing 4 native rumen microorganisms on milk production and efficiency of dairy cows. Mid-lactation Holstein cows (n = 90; 43% primiparous; 92 ± 23 DIM) averaging 45 kg milk/d were studied in 2 time cohorts. Cows were fed a basal diet containing 43% forage, 29% NDF, 29% starch, and 18% CP. After 14 d, they were blocked by parity, DIM, and energy-corrected milk (ECM) per metabolic BW. Within block, cows were randomly assigned to 1 of 3 treatments which were top-dressed daily for the next 112 d onto the basal diet. Treatments were 150 g of ground corn mixed with 1) no live DFM (CON), 2) 5 g of a live DFM (Galaxis Frontier; G2), and 3) 5 g of DFM (Galaxis Frontier; G2P). G2 contained *Clostridium beijerinckii* at 1×10^7 cfu/d and *Pichia kudriavzevii, Ruminococcus bovis,* and *Butyrivibrio fibrisolvens* at 1×10^8 cfu/d. G2P was similar but with higher levels of *C. beijerinckii* at 4×10^7 cfu/d and *P. kudriavzevii* at 1×10^9 cfu/d. G2 and G2P are products of Native Microbials Inc. (San Diego, CA). Data were analyzed using PROC MIXED in SAS with pretreatment period as covariate for production. DFM did not alter yield of total milk, protein, or fat ($P > 0.2$), but slightly decreased gain of BW ($P = 0.02$) and body condition ($P = 0.05$) with no difference between G2 and G2P ($P = 0.7$). DFM tended to decrease dry matter intake (DMI; $P = 0.08$) and tended to improve feed efficiency ($P = 0.06$) (ECM/DMI). DFM did not alter digestibility of fiber, starch, protein, or fat and did not alter concentrations of glucose or nonesterified fatty acids but tended to decrease concentration of insulin in plasma averaged over a day ($P = 0.057$). DFM decreased somatic cell counts in milk ($P = 0.05$) with no difference between G2 and G2P. In conclusion, supplementation with DFM had little impact on mid-lactation production, but some trending improvements in feed efficiency were observed. The digestibilities of NDF and starch in our diet were relatively high (45% and 95%, respectively), which might explain the lack of improved performance.

Key Words: microbiome, feed additive, rumen

1439 Rumen endomicrobials improve lactation when supplemented during the periparturient period and mid-lactation in Holstein dairy cows. M. Bulnes*[1], G Mendizabal[1], J. Bonilla[1], M Suazo[1,3], T. C. Michelotti[1,2], A. Paz[1], G. Begalli[1,4], A. F. Souza[1,4], J. Lefler[5], C. Marotz[5], M. E. Uddin[1], and J. Osorio[1], [1]*South Dakota State University, Brookings, SD,* [2]*University of Minnesota, Twin Cities, MN,* [3]*Texas Tech University, Lubbock, TX,* [4]*University of Lavras, Lavras, MG, Brazil,* [5]*Native Microbials Inc., San Diego, CA.*

Endomicrobials (EM) are native rumen microbial organisms that have been selected and cultured with the purpose of improving rumen function and feed efficiency in dairy cattle. This study evaluated the effects of a novel EM [Galaxis Frontier (GF); Native Microbials, Inc., California, USA] composed of a curated group of rumen microorganisms present in and originally isolated from high-performing dairy cows. Fifty-six Holstein dairy cows were enrolled at −21 d relative to calving and remained on the experiment until 100 d in milk (DIM). Cows were used in a randomized complete block design, where expected calving date, parity, and previous lactation milk yield for multiparous or genetic merit for primiparous cows were used as blocking factors. All cows received the same close-up diet from −21 DIM until calving (1.29 Mcal/kg DM and 10.8% CP) and lactation diet from calving to 100 DIM (1.67 Mcal/kg DM and 15.3% CP). At −21 DIM, cows were randomly assigned to a basal diet plus 150 g/d of ground corn (CON; n = 29) or a basal diet plus 150 g/d and 5 g/d GF (n = 27) for the remainder of the trial. Additional samples collected during this trial include blood and rumen fluid. Blood samples are being analyzed for inflammation and oxidative stress biomarkers, while ammonia, VFA, and microbiome composition in rumen fluid. Data were analyzed using the MIXED procedure of SAS. There was a trend ($P = 0.08$) for increased milk yield (+2.64 kg/d) for cows fed GF than CON during mid-lactation (31 to 100 DIM). Although DMI was not affected by treatment, GF cows tended ($P = 0.10$) to have a greater feed efficiency (+0.11, milk/DMI) in early lactation (0 to 30

DIM). There was a treatment × time interaction ($P < 0.01$) for milk fat and protein %, where milk fat % was lower ($P < 0.01$) in GF cows than CON at wk 11. Milk protein % was greater ($P = 0.04$) in GF cows than CON at wk 1, while lower ($P \leq 0.04$) in GF cows than CON at wk 9 and 13. These results suggest that peripartal supplementation with GF will promote a better lactation performance, partially explaining improvements in feed efficiency.

Key Words: rumen endomicrobials, lactation performance, transition cows

1440 Effects of heat stress conditions and dietary organic acid and pure botanical supplementation on gastrointestinal permeability and plasma trimethylamine *N*-oxide concentrations in lactating cows. A. B. P. Fontoura*[1], A. Javaid[1], V. Sáinz de la Maza-Escolà[1,2], N. S. Salandy[1,3], S. L. Fubini[1], E. Grilli[2], and J. W. McFadden[1], [1]*Cornell University, Ithaca, NY,* [2]*Università di Bologna, Bologna, Italy,* [3]*Tuskegee University, Tuskegee, AL.*

In dairy cows, heat stress may develop with a modified gut microbiome, thus altering plasma concentrations of microbial-derived trimethylamine *N*-oxide (TMAO) with a concomitant change in gastrointestinal permeability (GP). Dietary organic acid and pure botanical (OA/PB) feeding may prevent these outcomes. Forty-eight Holstein cows (208 ± 4.65 d in milk [mean ± SD], 3.0 ± 0.42 lactations, 122 ± 4.92 d pregnant) were enrolled in a study with a completely randomized design. Following a 7-d acclimation in thermoneutral conditions (temperature-humidity index [THI] 68), cows were assigned to 1 of 4 groups (n = 12/group): thermoneutral conditions (TN-Con), heat stress (HS) conditions (HS-Con; diurnal THI 74 to 82), thermoneutral conditions pair-fed to match HS-Con (TN-PF), or HS fed OA/PB (HS-OAPB; 75 mg/kg of body weight; 25% citric acid, 16.7% sorbic acid, 1.7% thymol, 1.0% vanillin, and 55.6% triglyceride; AviPlusR, Vetagro, Italy) for 14 d. Cows were fed a corn silage based total mixed ration top-dressed without (triglyceride only) or with OA/PB. An oral Cr-EDTA challenge was performed to measure GP on d 3 and 13. Blood was collected on d −1, 3, and 14. Plasma Cr and TMAO were quantified. Data were analyzed using a mixed model including fixed effects of treatment, time, and their interaction. Contrasts included HS-Con vs. TN-Con, HS-Con vs. TN-PF, and HS-Con vs. HS-OAPB. HS-Con had greater plasma Cr area under the curve (AUC; $P = 0.05$) and tendency for greater Cr AUC ($P = 0.12$) on d 3, relative to TN-Con and TN-PF, respectively. HS-Con had similar plasma Cr AUC on d 13, relative to TN-PF and TN-Con. TN-PF tended to have greater plasma Cr concentrations from h 12 to 24 post bolus on d 13, relative to TN-Con (Treatment × Time, $P = 0.13$). HS-Con had lower plasma TMAO concentrations on d 3 and 14, relative to TN-Con or TN-PF ($P < 0.01$). HS-OAPB plasma Cr AUC or TMAO concentrations were not different from HS-Con on d 3 or 14. We conclude that heat stress increases GP in cows independent of changes in intake or OA/PB feeding, and decreases in plasma TMAO are suggestive of a modified gut microbiome during HS.

Key Words: heat stress, leaky gut, TMAO

1441 Effects of dietary betaine supplementation and partial rumen content transplantation on clinical signs of hyperthermia and milk production in heat-stressed Holstein cows. A. Javaid*[1], A. R. Gonzalez[2], J. W. McFadden[1], and D. E. Rico[3], [1]*Cornell University, Ithaca, NY,* [2]*Université Laval, Québec, QC, Canada,* [3]*CRSAD, Deschambault, QC, Canada.*

Heat stress can alter the rumen microbiome and fermentation in cows; which may be modified by dietary betaine supplementation. Twelve rumen-cannulated multiparous Holstein cows (39 ± 6.4 kg milk/d; 82 ± 27 d in milk [DIM]) were used in a split-plot design testing the effects of betaine and partial rumen content transplantation (PRCT) on cow performance during heat stress. The main plot was the level of dietary betaine supplementation (CON: unsupplemented; or BET: 100 g/d intra-ruminal betaine hydrochloride 95%; AB Vista, Canada). Within each plot, cows were randomly assigned to the following treatments 1) heat stress (HS), 2) thermoneutral pair-feeding (TNPF), or 3) HS with PRCT (HS+PRCT; 25% replacement of rumen contents from 4 donor cows fed ad libitum in thermoneutrality; d 8–14) in a replicated 3 × 3 Latin square design with 14-d periods. A mock transplantation was performed in HS and TNPF cows, as a handling control. Dry matter intake (DMI) and rectal temperature were recorded daily, and water intake and respiratory rates were determined on d 0, 3, 5, 7, 10 and 13. Milk samples were collected on d 0, 3, 7, 10 and 13. The statistical model included the random effects of cow and period, and the fixed effects of plot, treatment, day, and their interactions. No block or interaction effects were detected for any variable. Respiration rates, rectal temperatures, and water intakes were increased by 52%, 28% and 6%, respectively, in HS relative to TNPF ($P < 0.01$), but were not different between HS and HS+PRCT. Milk yields tended to be 23% lower in HS compared with TNPF cows (20.9 ± 1.4 vs. 25.1 ± 1.4 kg/d; $P = 0.06$) but were not different between HS and HS-PRCT (20.9 ± 1.4 vs. 16.5 ± 1.6 kg/d). Heat stress reduced the yield of milk protein ($P = 0.02$) by 22%, relative to TNPF (2.8 ± 0.1 vs. 3.0 ± 0.8 kg/d). However, milk protein yield was not different between HS and HS+PRCT. The yield of milk fat was not affected by treatment. We conclude that dietary betaine supplementation and PRCT had a limited ability to prevent the effects of heat stress on milk production in cows. Supported by FFAR.

Key Words: betaine, heat stress, ruminal microbiota

1442 Evaluating methane mitigation by organic-certified feed additives within continuous culture. B. A. Wenner*[1], K. E. Mitchell[1], G. Praisler[1], S. Kienzle[1], J. S. Velez[2], and P. S. Yoder[3], [1]*The Ohio State University, Department of Animal Sciences, Columbus, OH,* [2]*Aurora Organic Dairy, Boulder, CO,* [3]*Perdue AgriBusiness, Salisbury, MD.*

Sustainability is interwoven with consumer expectations of organic agriculture yet there are limited independently validated strategies for methane (CH_4) mitigation for organic dairy systems. Thus, our objective was to compare 2 organically certified feed additives for CH_4 inhibition and one feed additive pending approval. We hypothesized that each would decrease CH_4 production in continuous culture when compared with a control diet. Using dual-flow continuous culture fermenters (DFCC) fitted for CH_4 and hydrogen sampling, 4 treatments were arranged in a 4 × 4 Latin square design. Treatments were a negative control (CON, 60:40 concentrate:orchardgrass pellet mix, 17.1% CP, 33.0% NDF, 20.1% ADF, and 27.1% starch) fed twice daily for a total of 80 g/d DM, CON plus kelp seaweed (KELP) at 1.7 g/d, CON plus essential oils (EO) at 3 mg/d, and CON plus biochar (CHAR) at 1.6 g/d. All dosages were calculated based on previous data and supplier recommendations scaled to DFCC functional volume. Experimental periods included 7 d adaptation and 4 d sampling (11 d total). Buffer and solids dilution rates were 7%/hr and 5%/hr, respectively. The statistical model included fixed effect of treatment and random effects of fermenter and period. Gas production data were measured by feeding, thus, analysis included a repeated effect of feeding and hourly VFA

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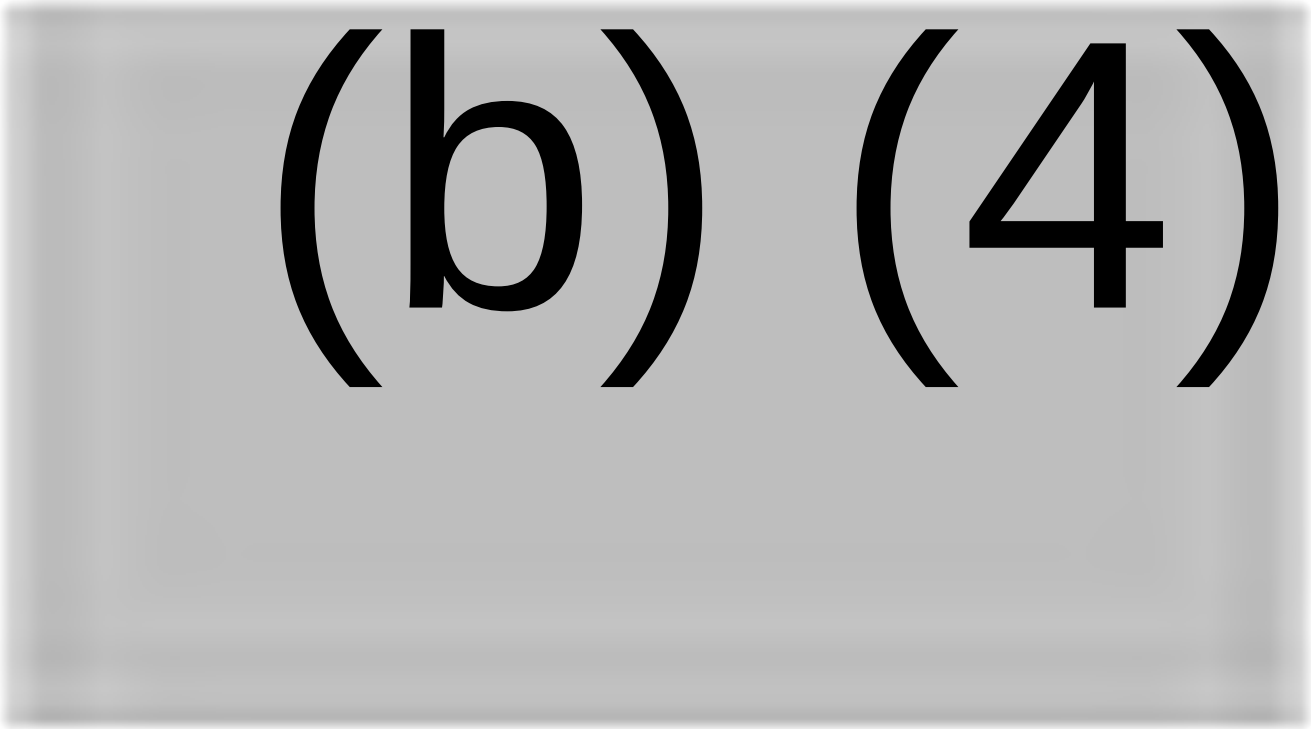
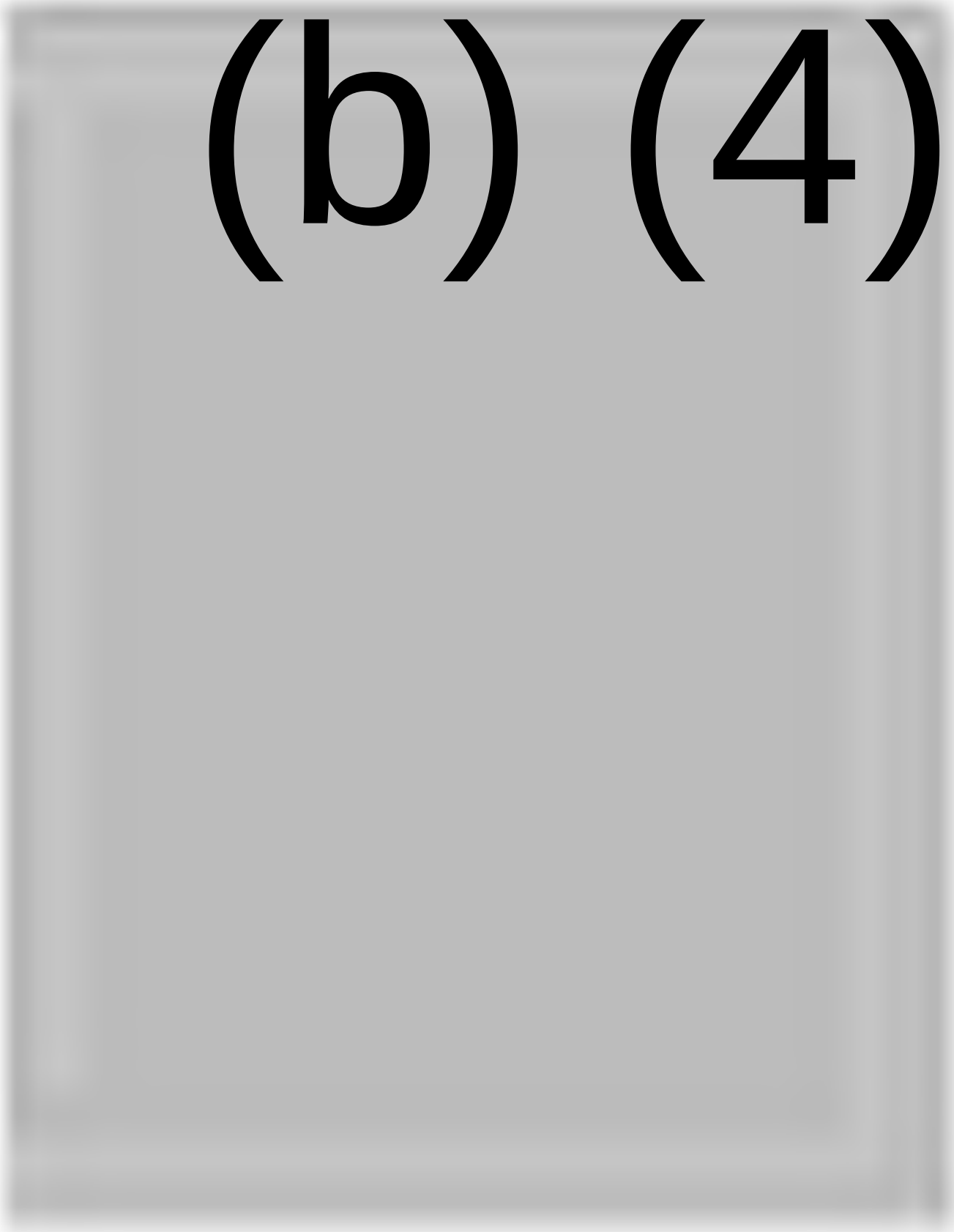
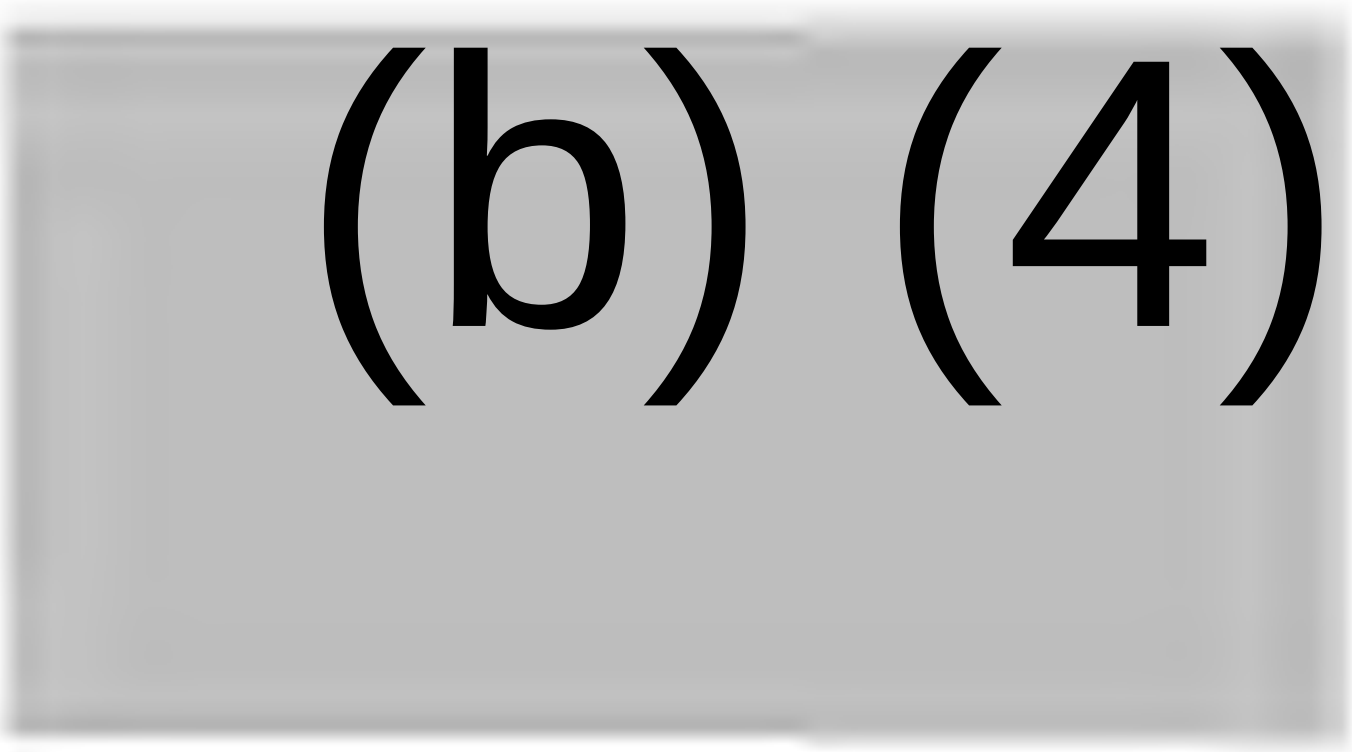


ABSTRACT

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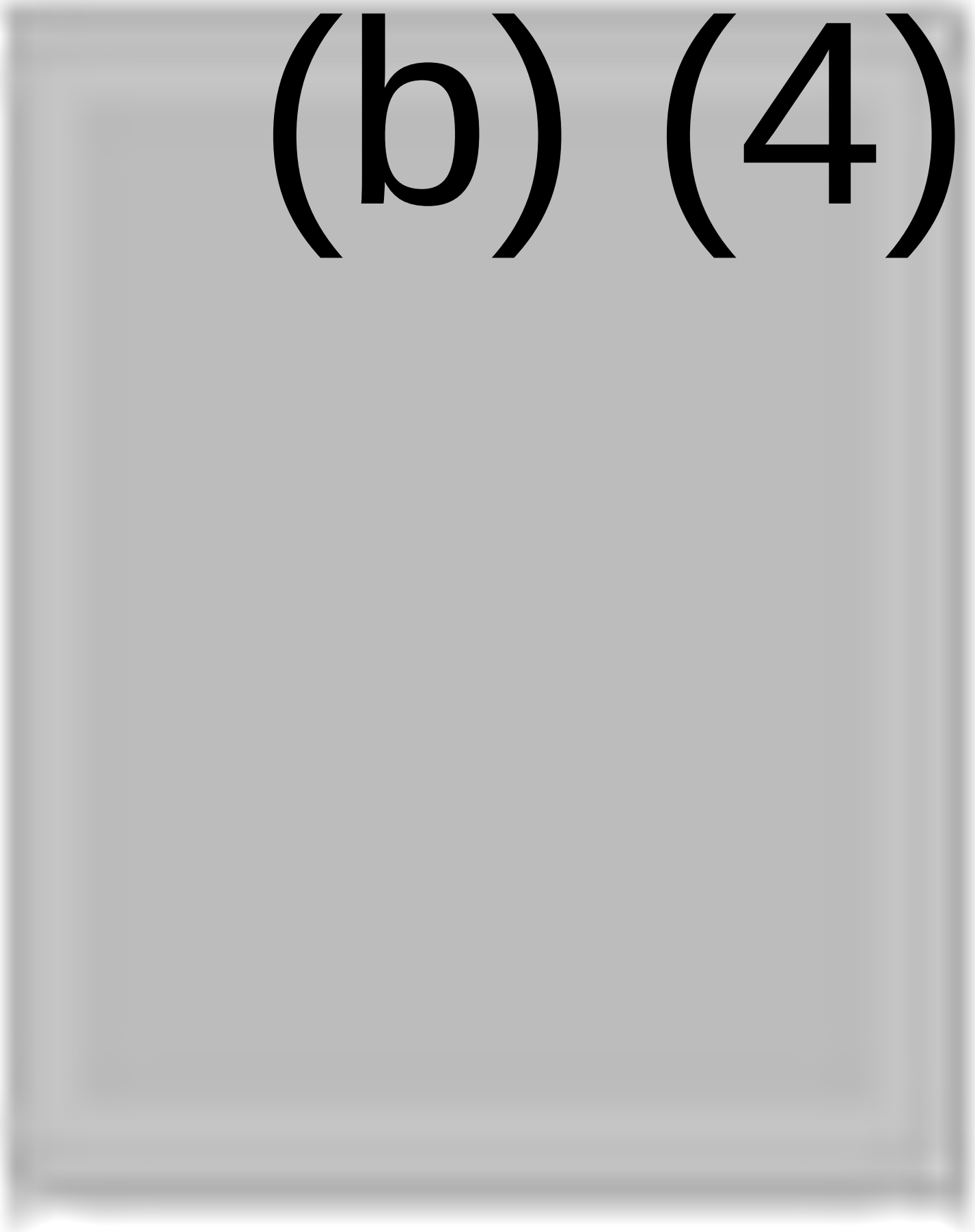
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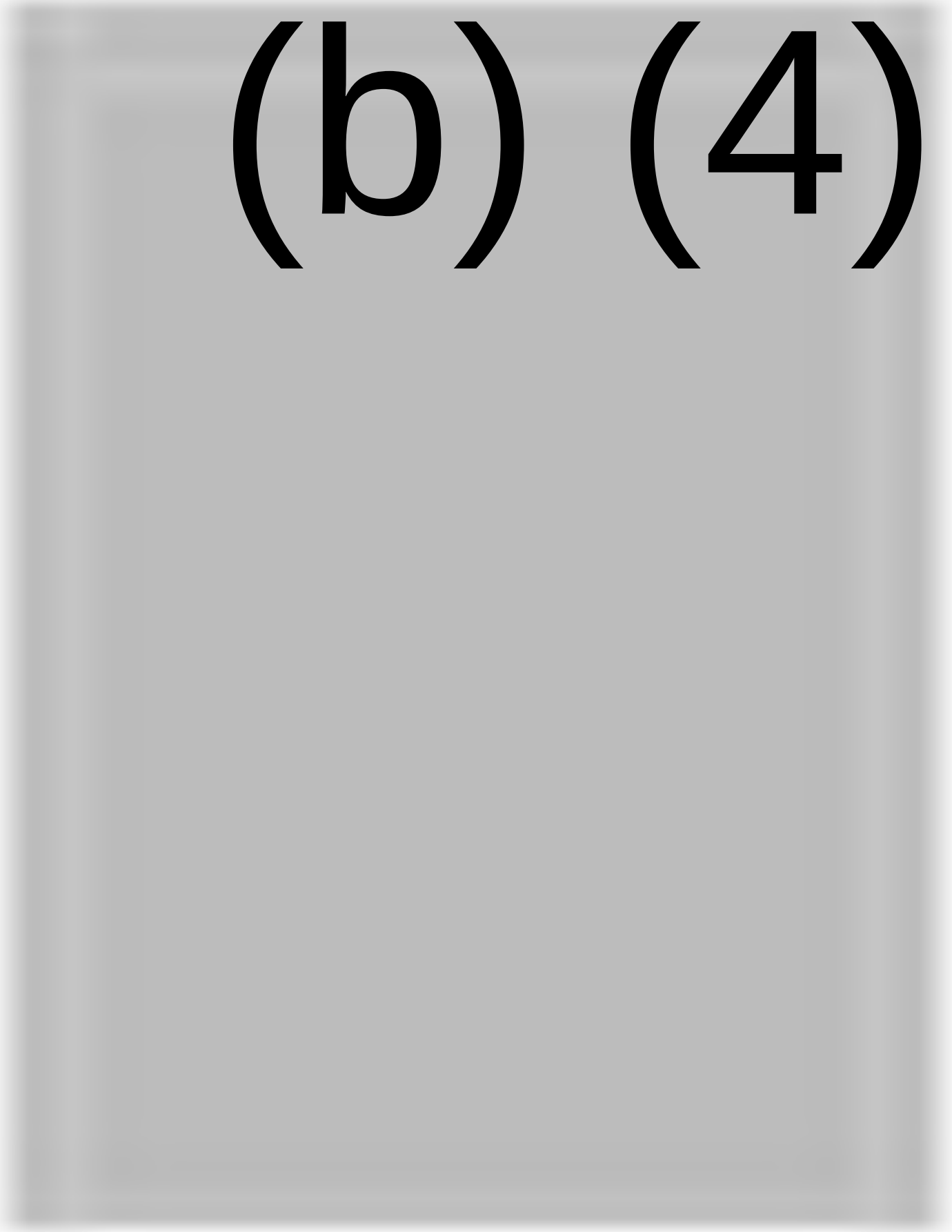
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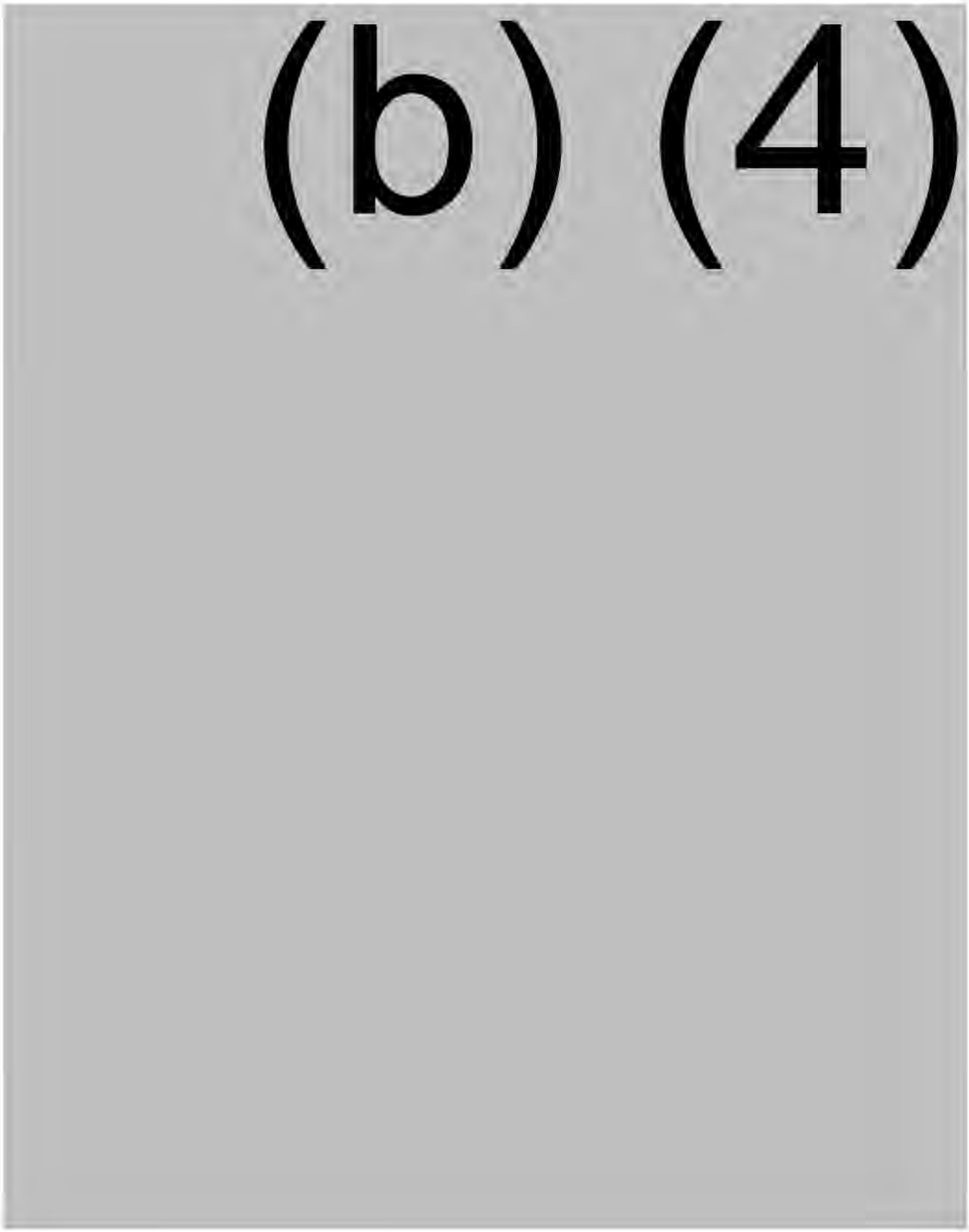
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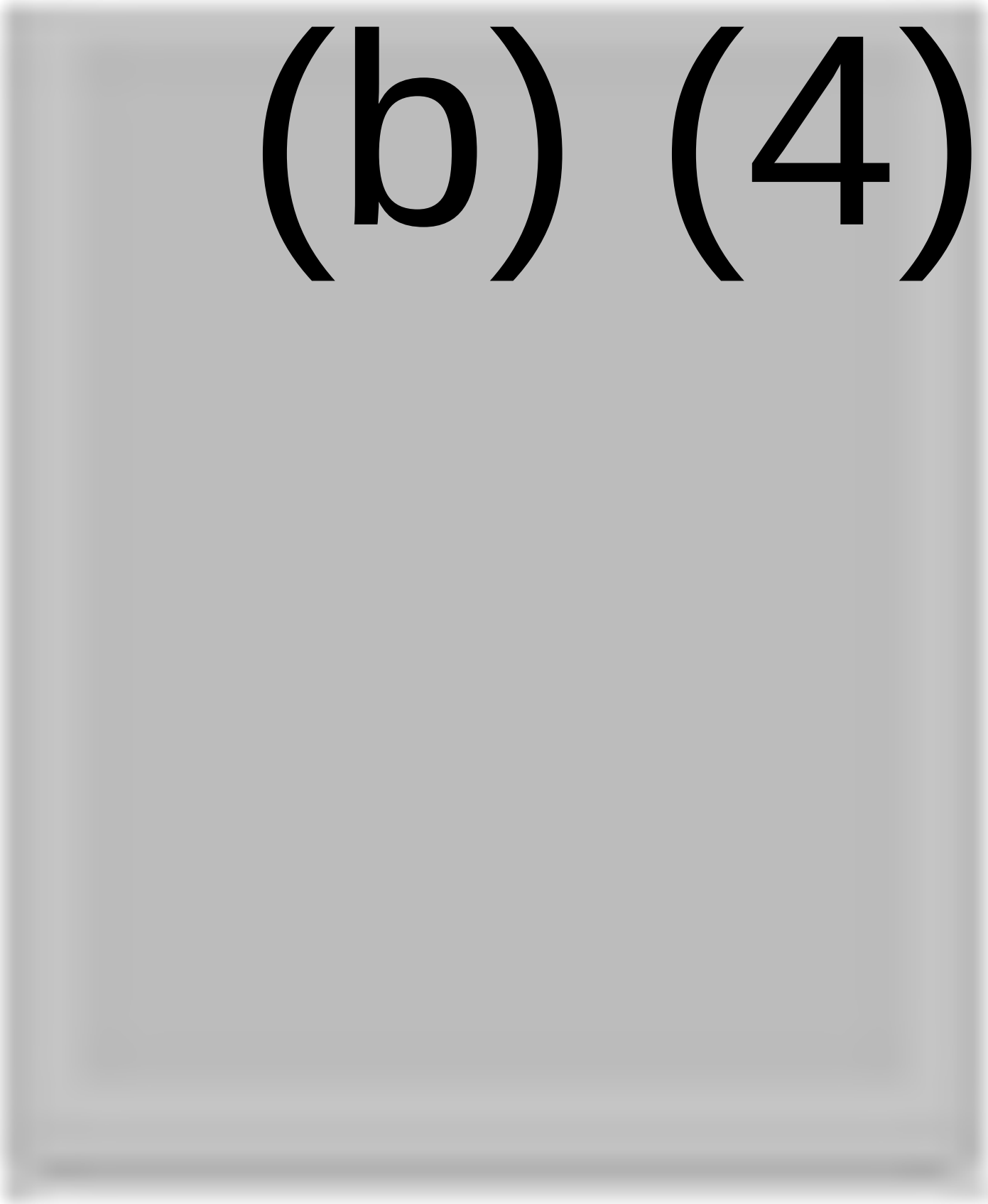
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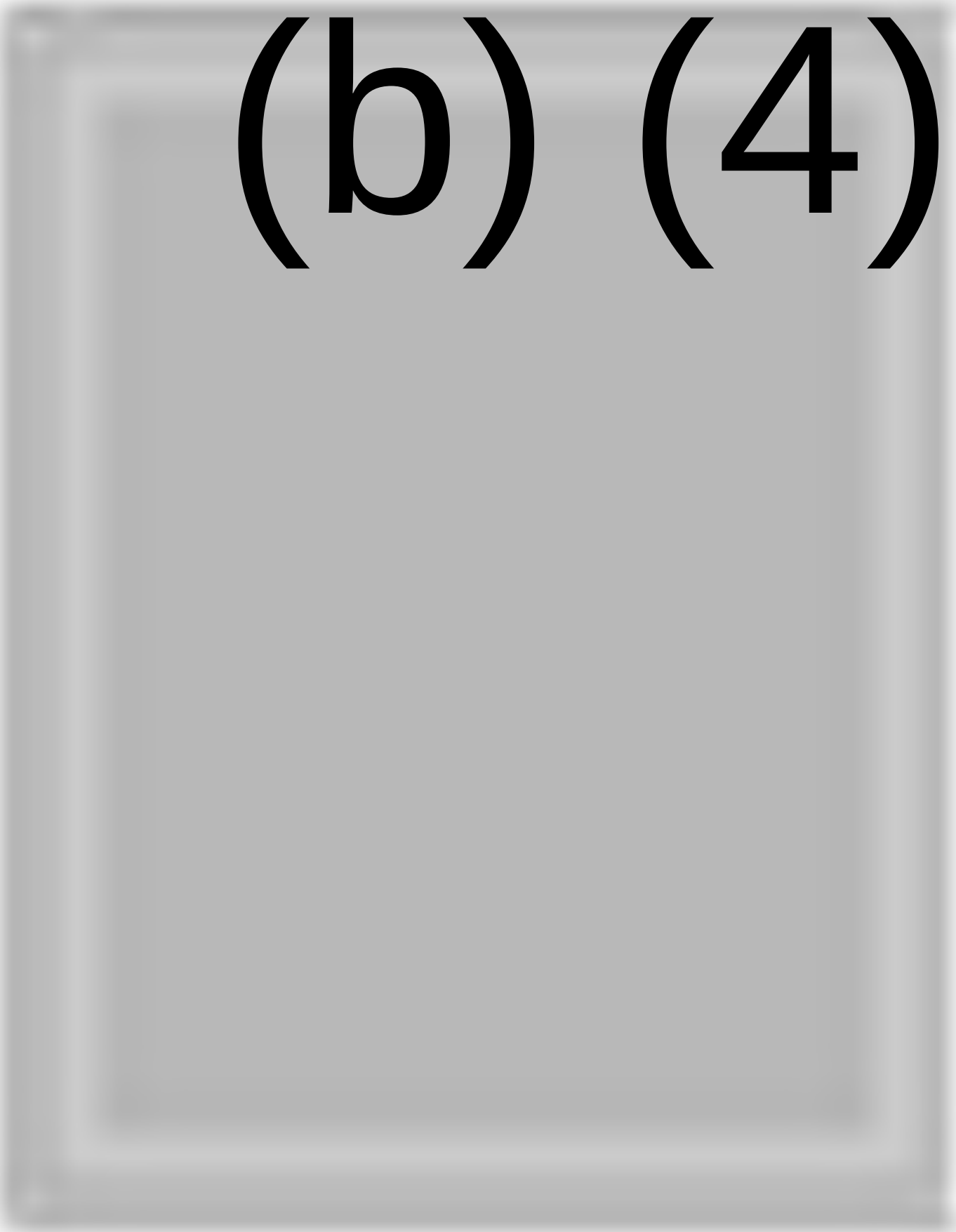
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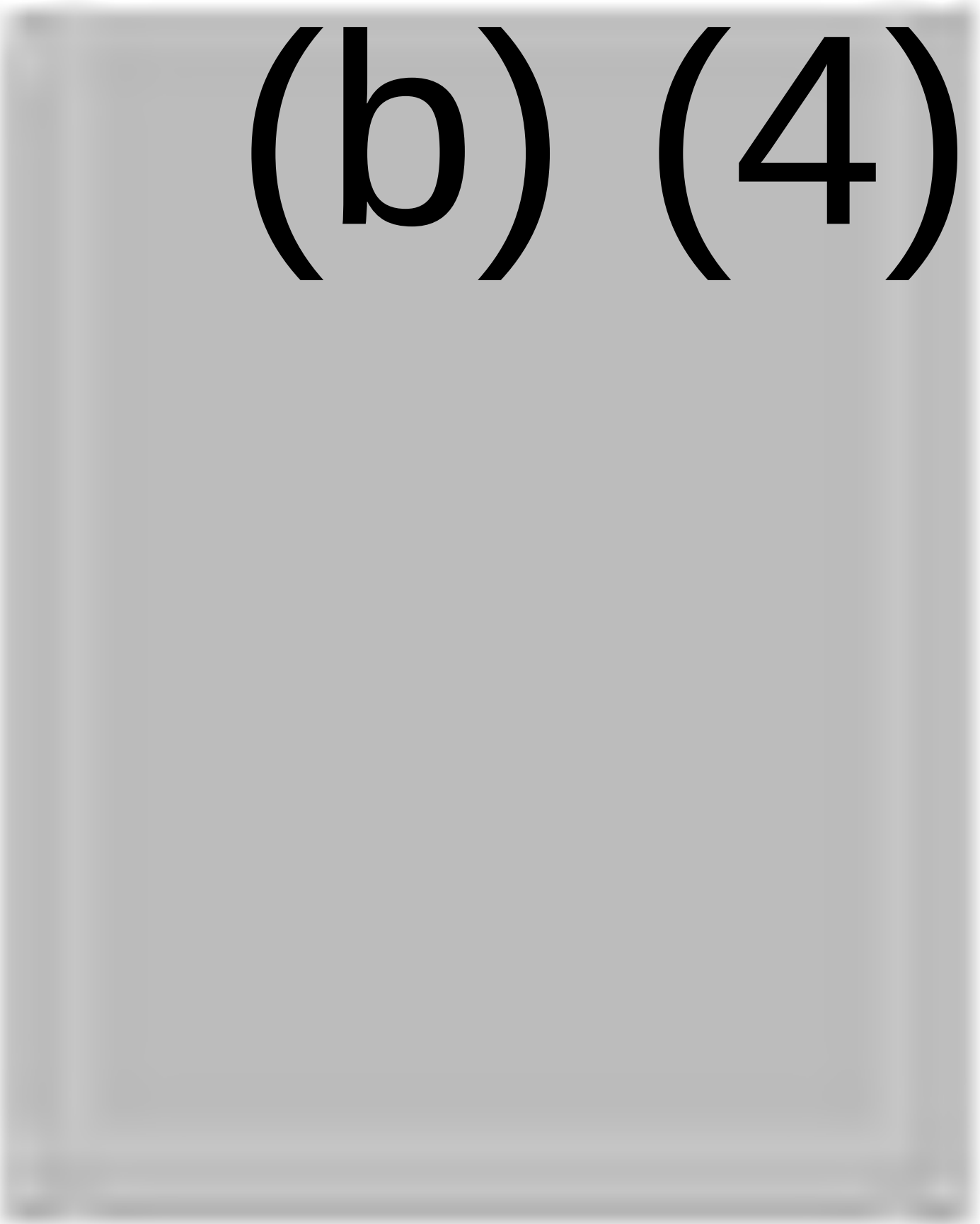
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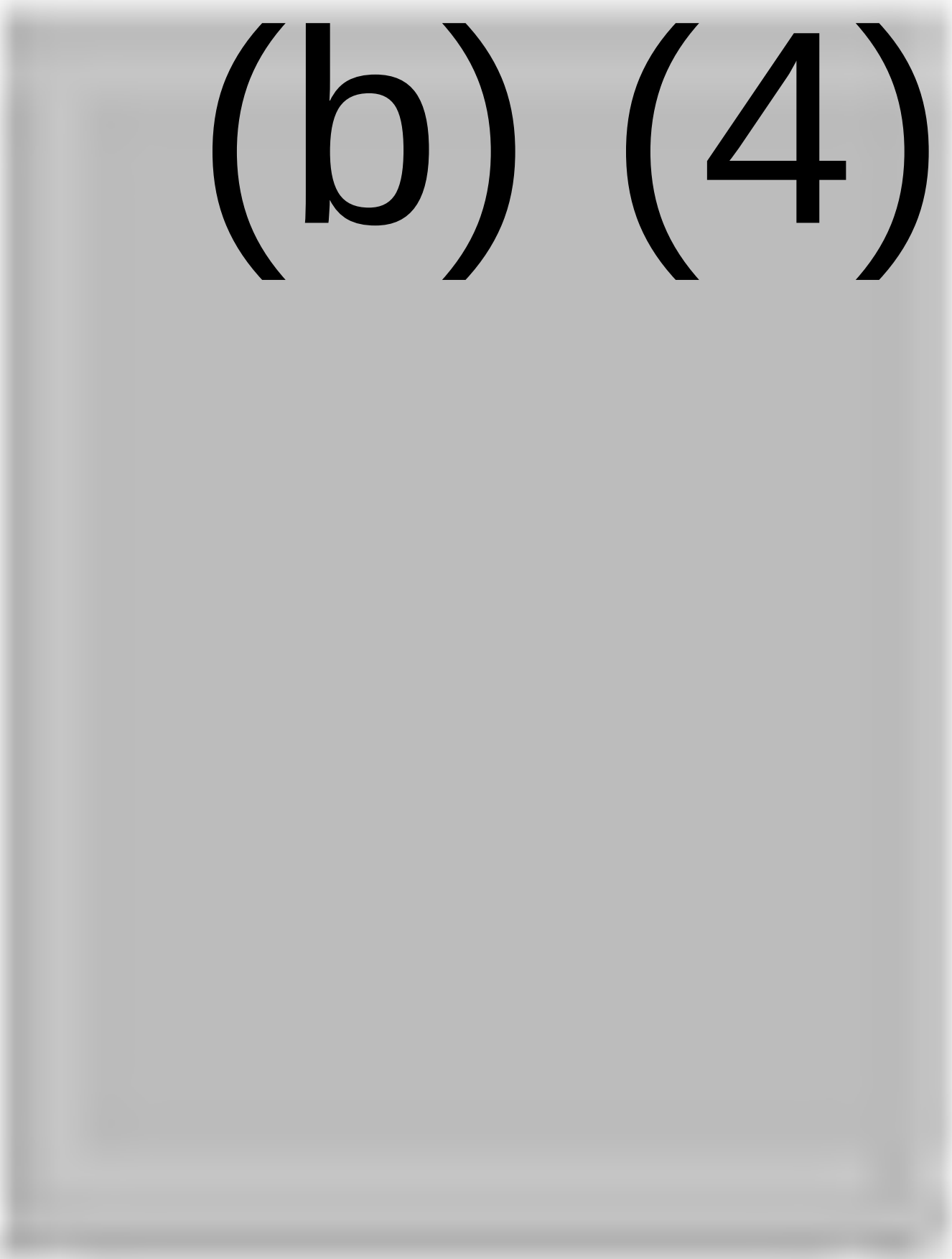
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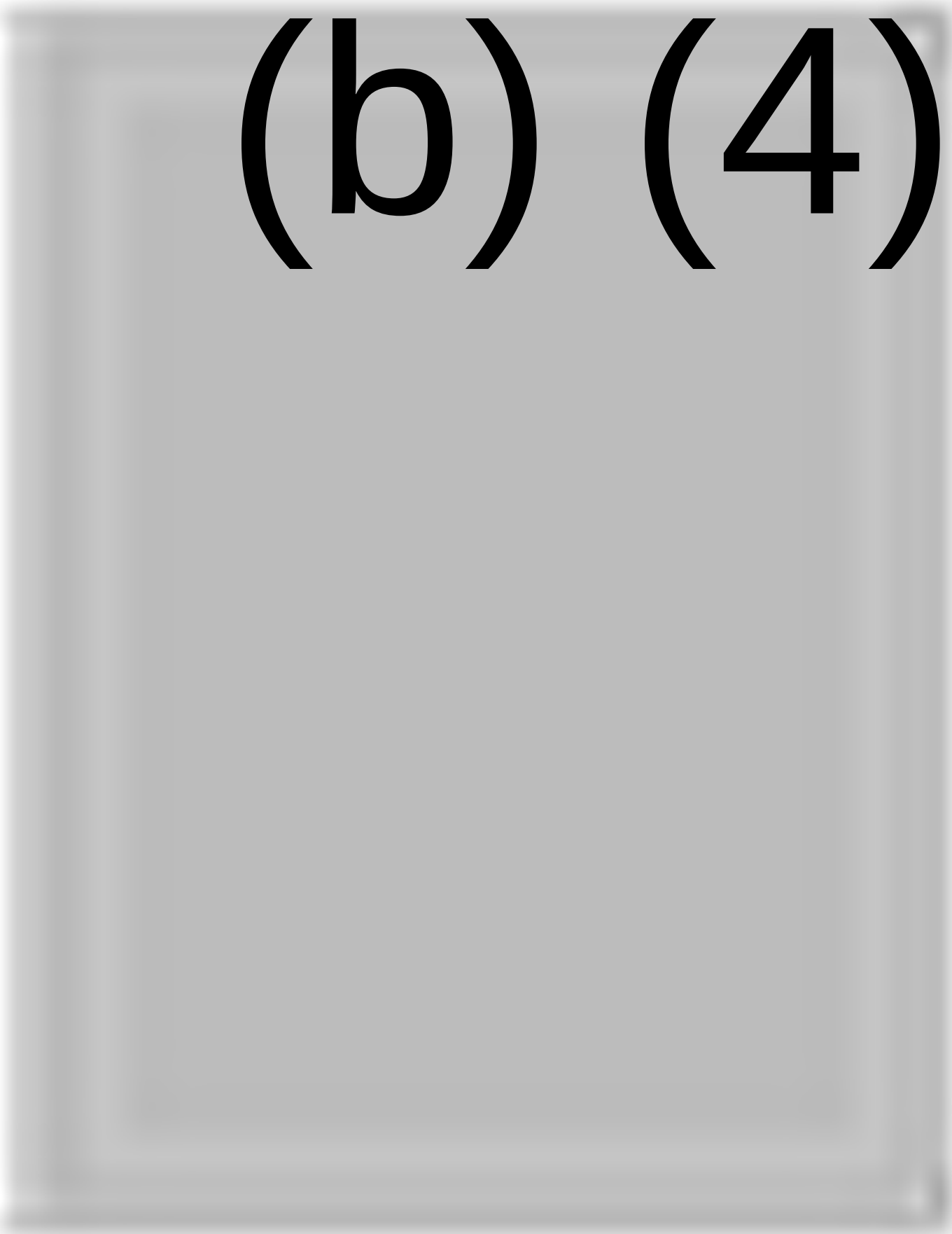
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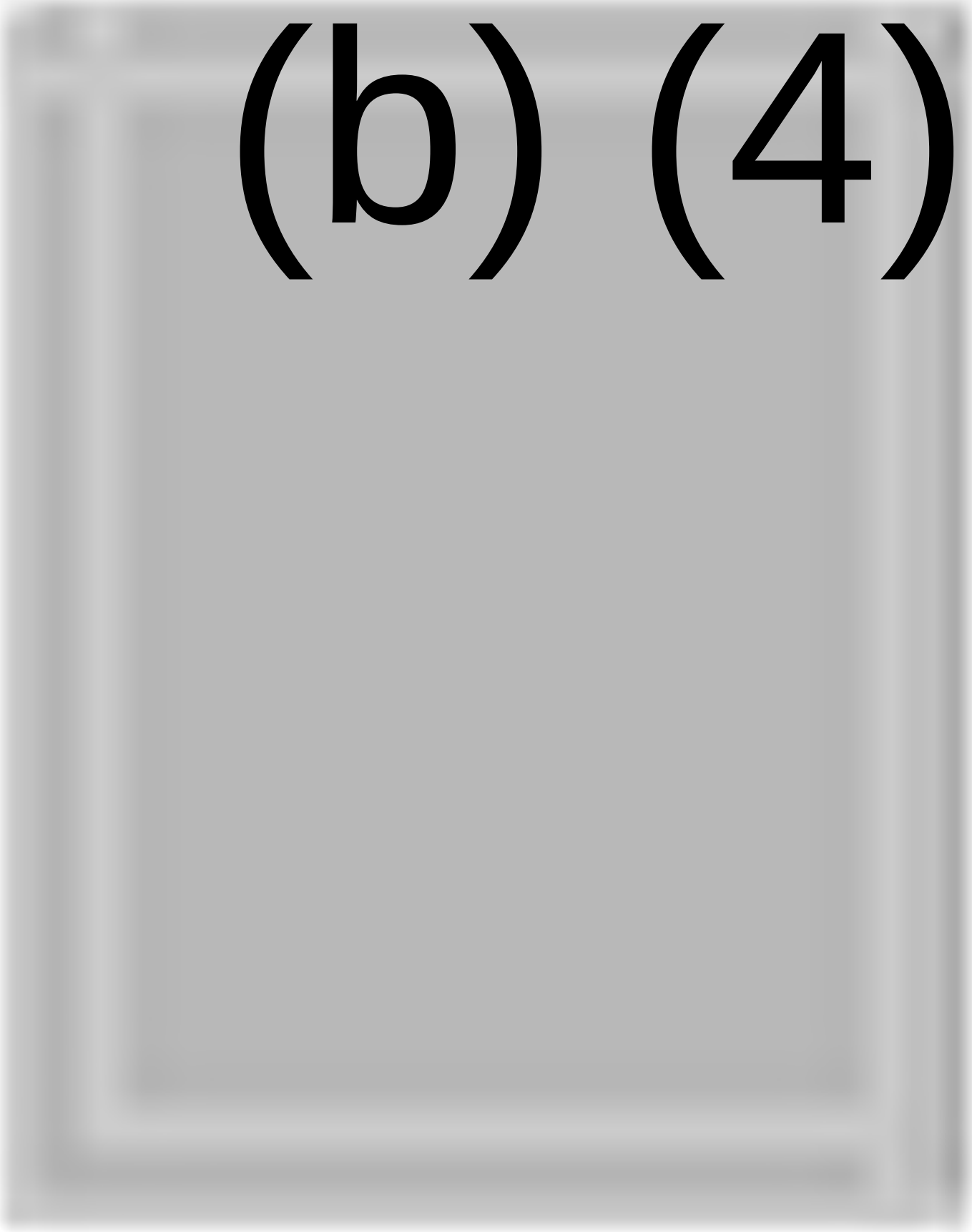
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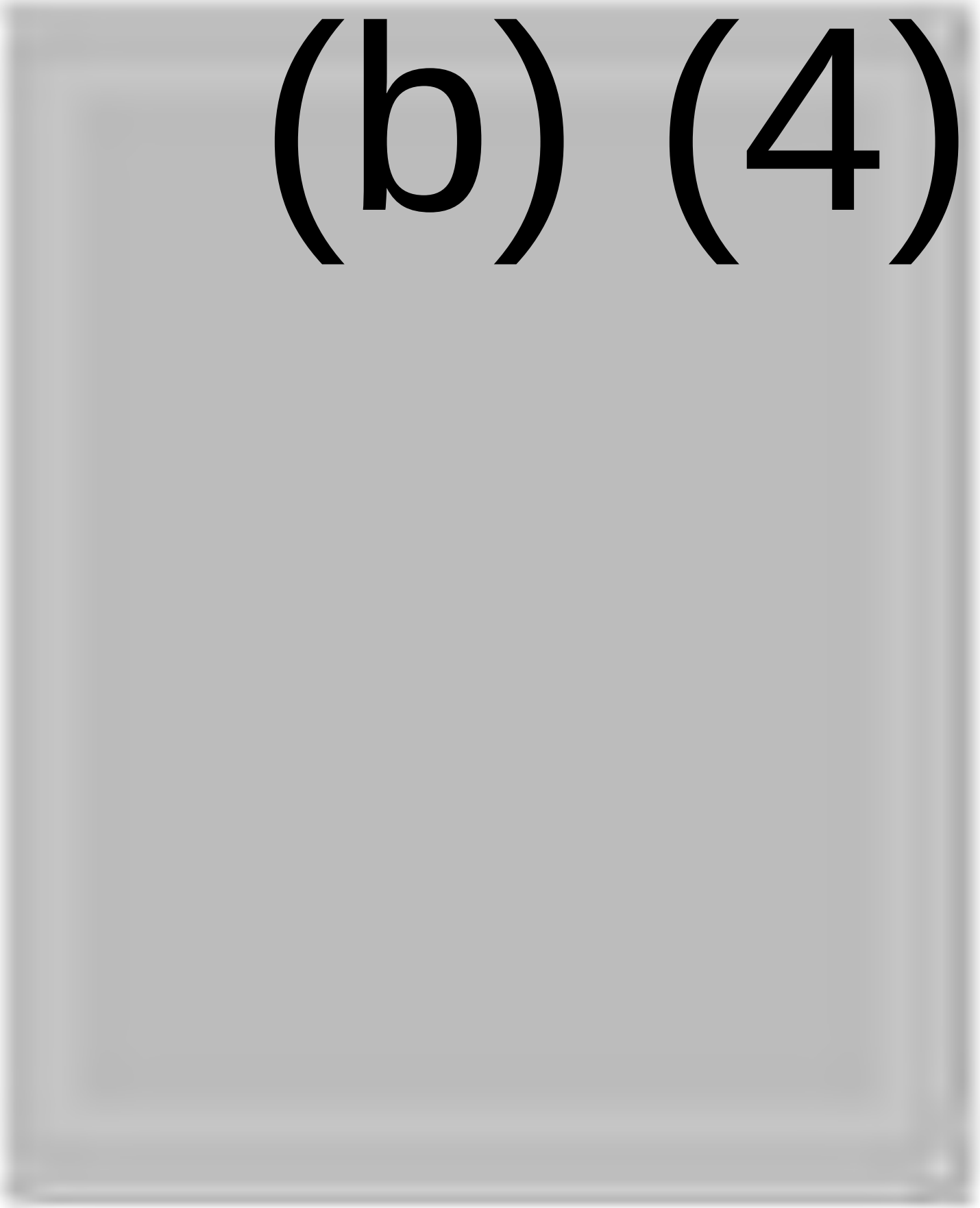
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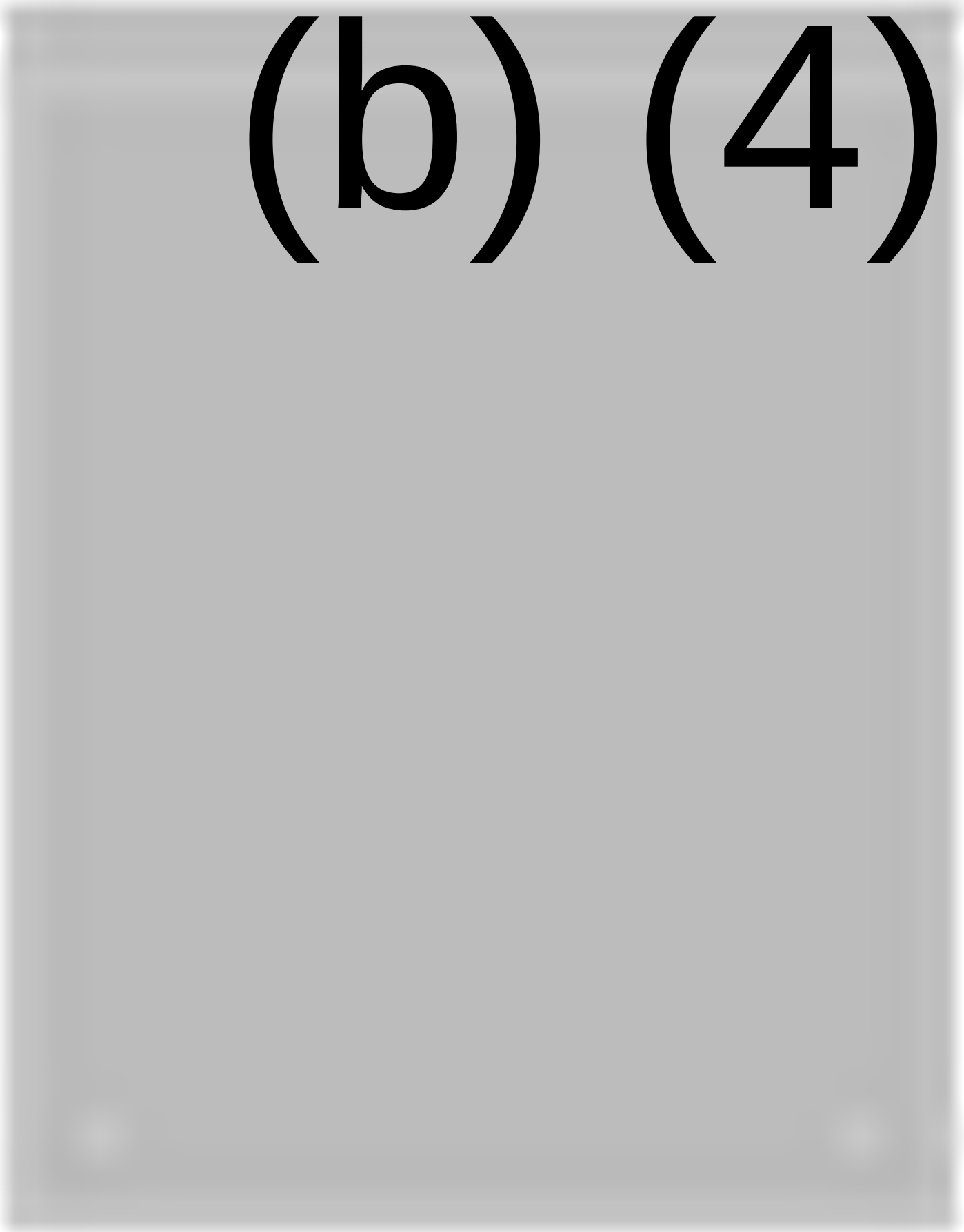
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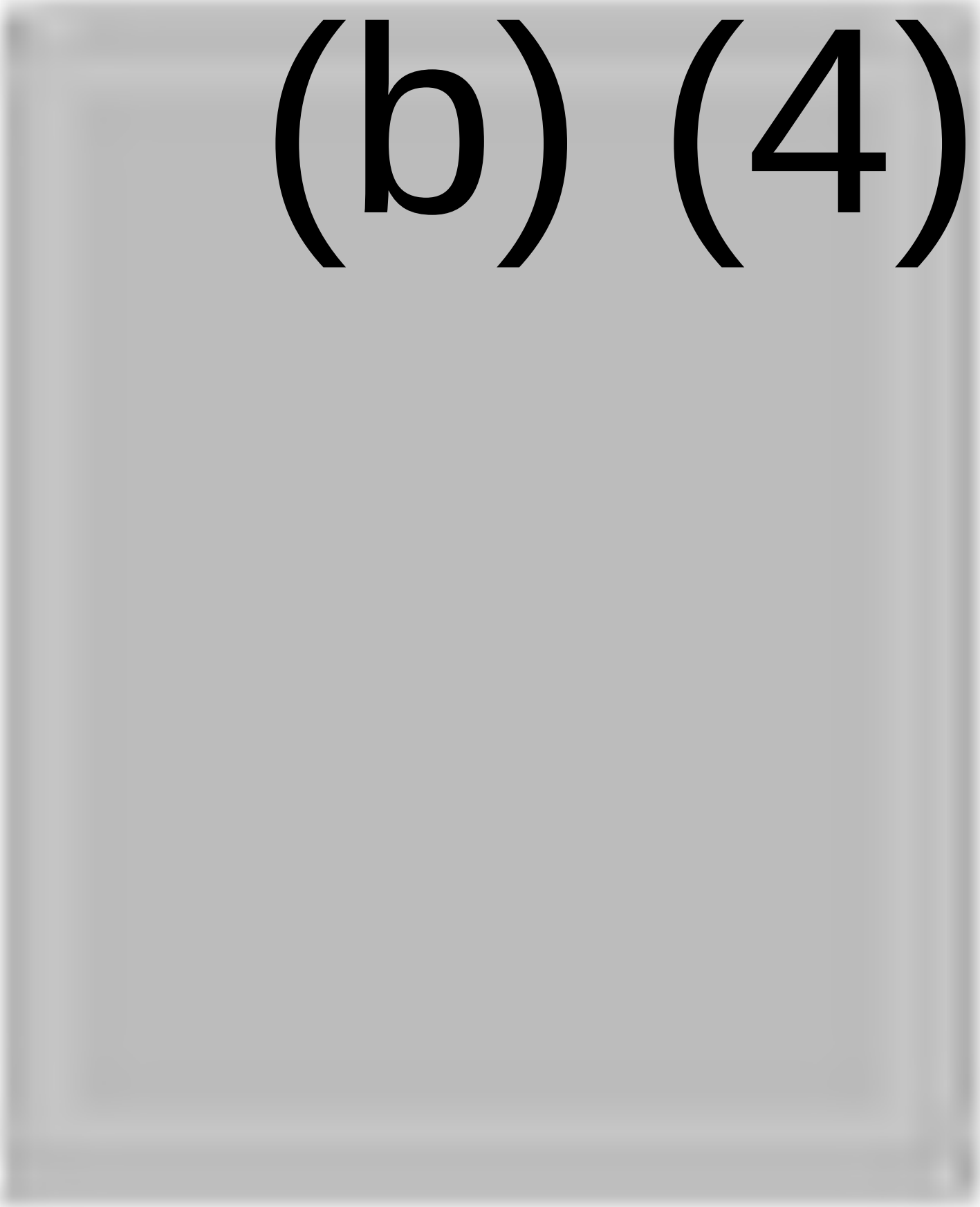
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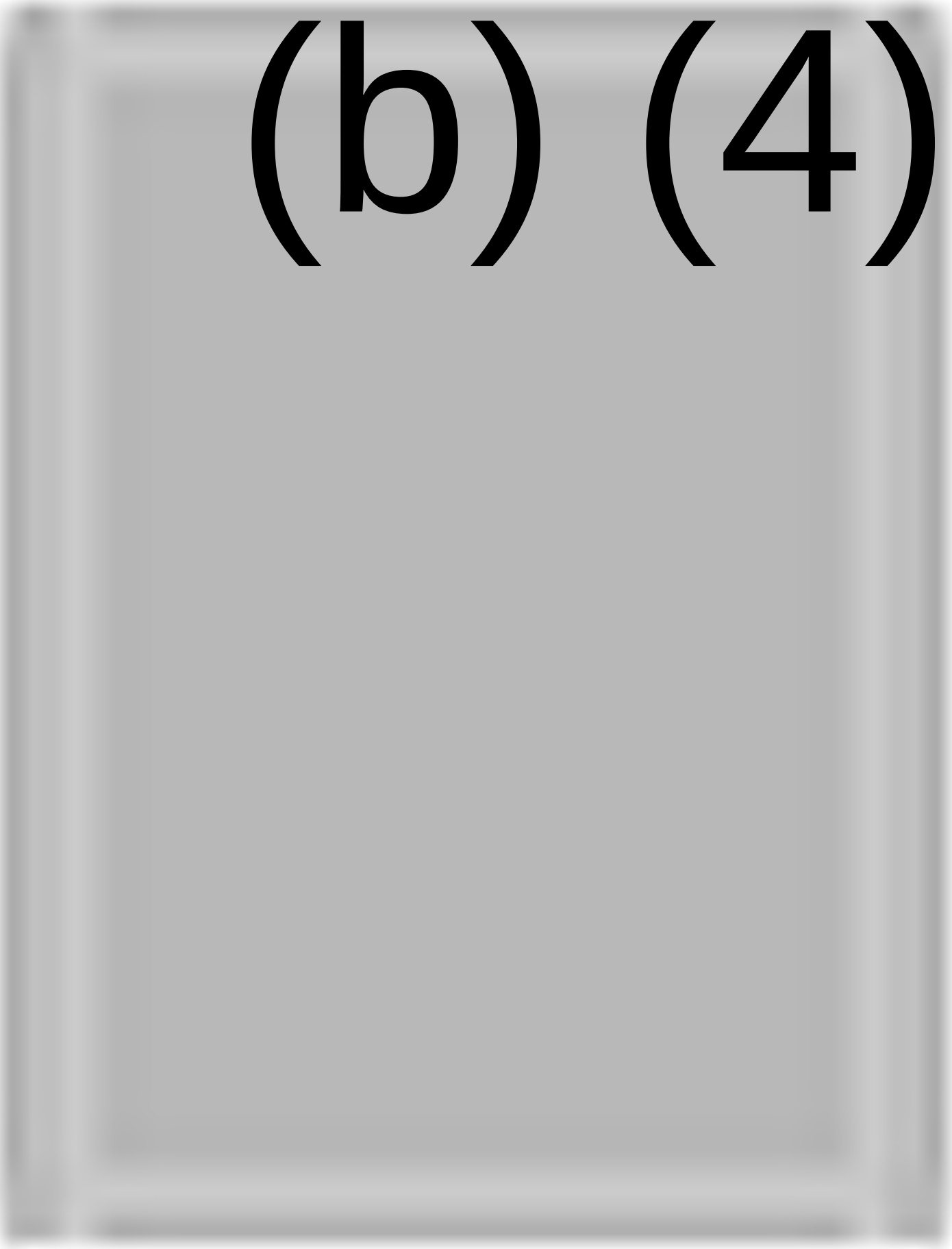
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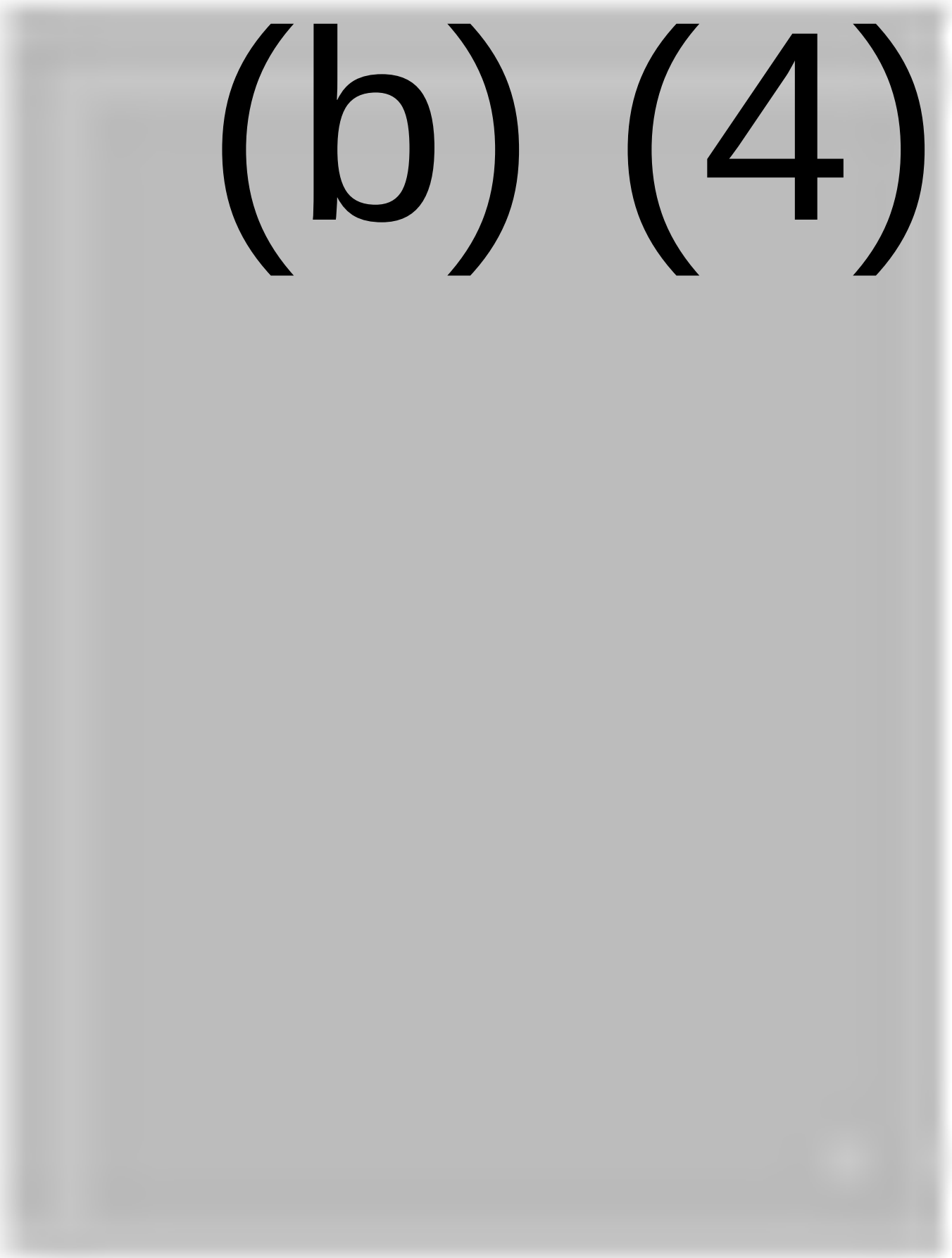
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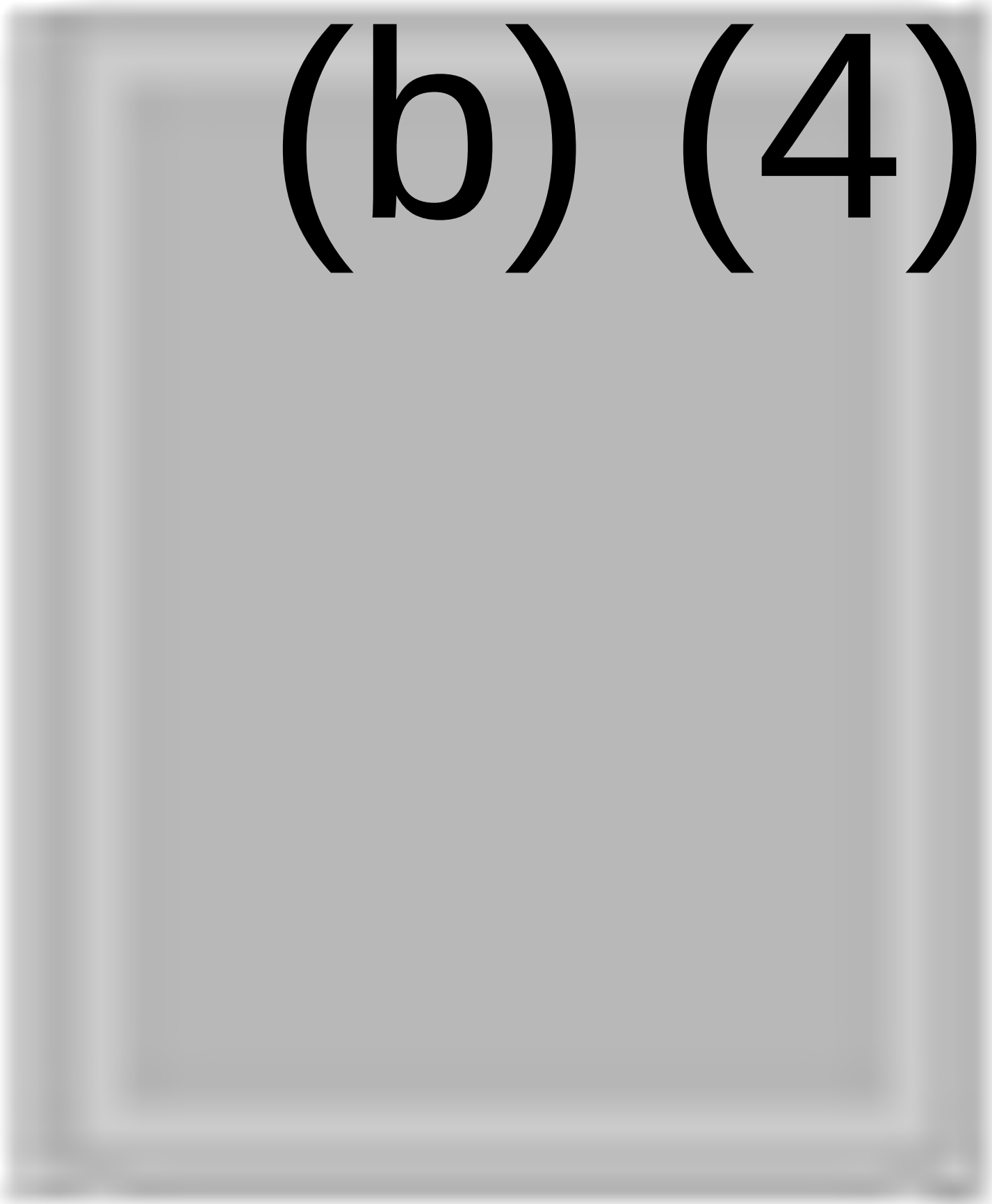
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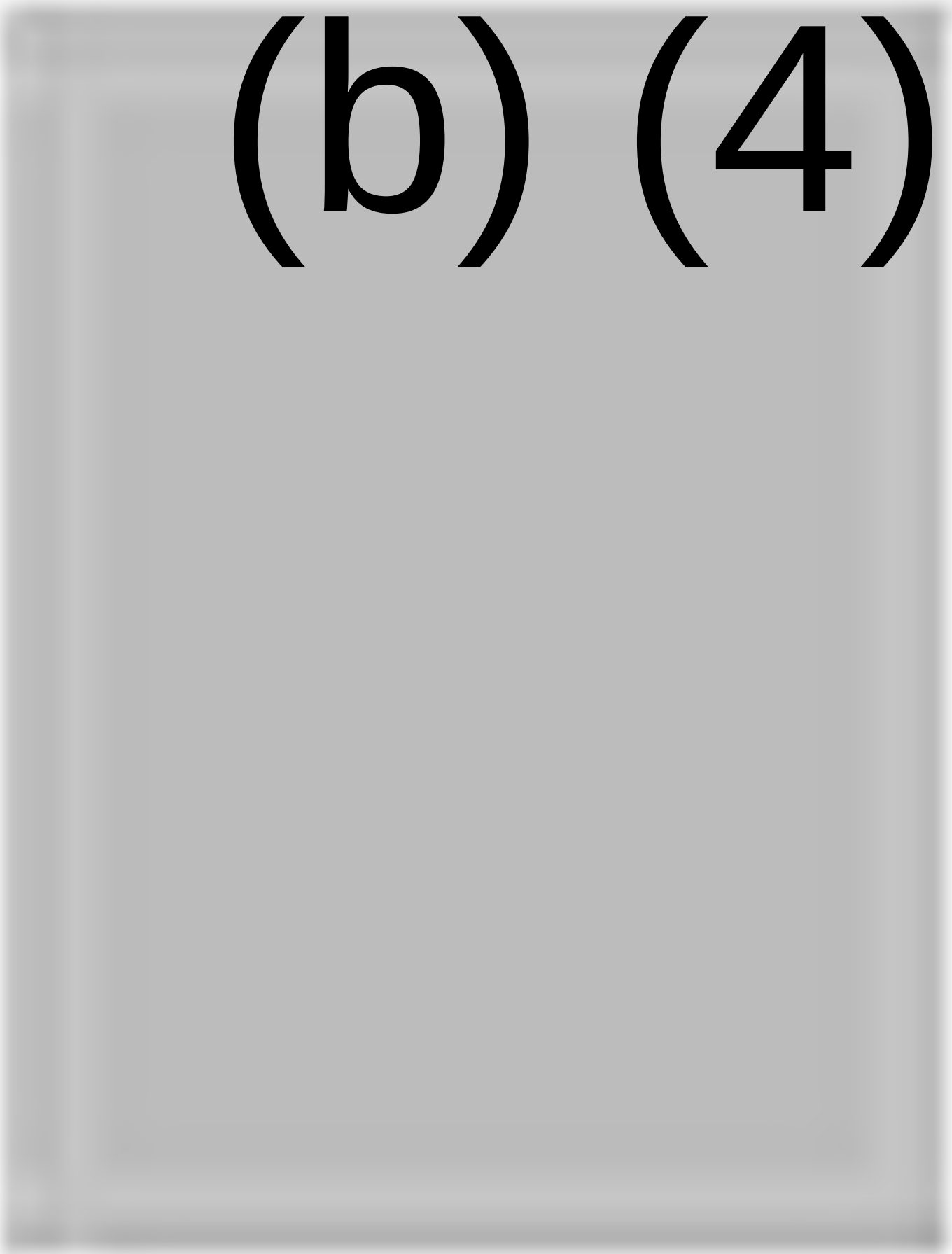
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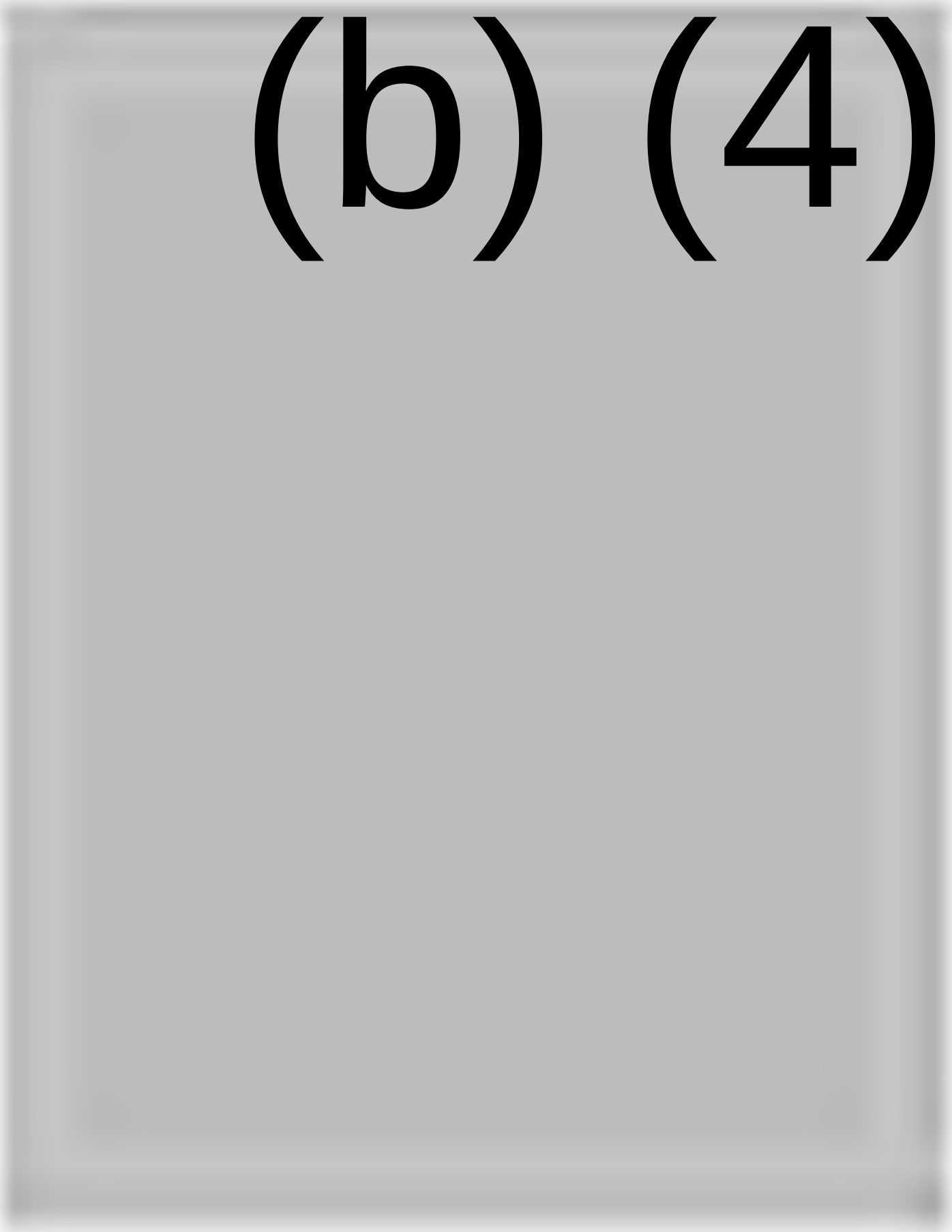
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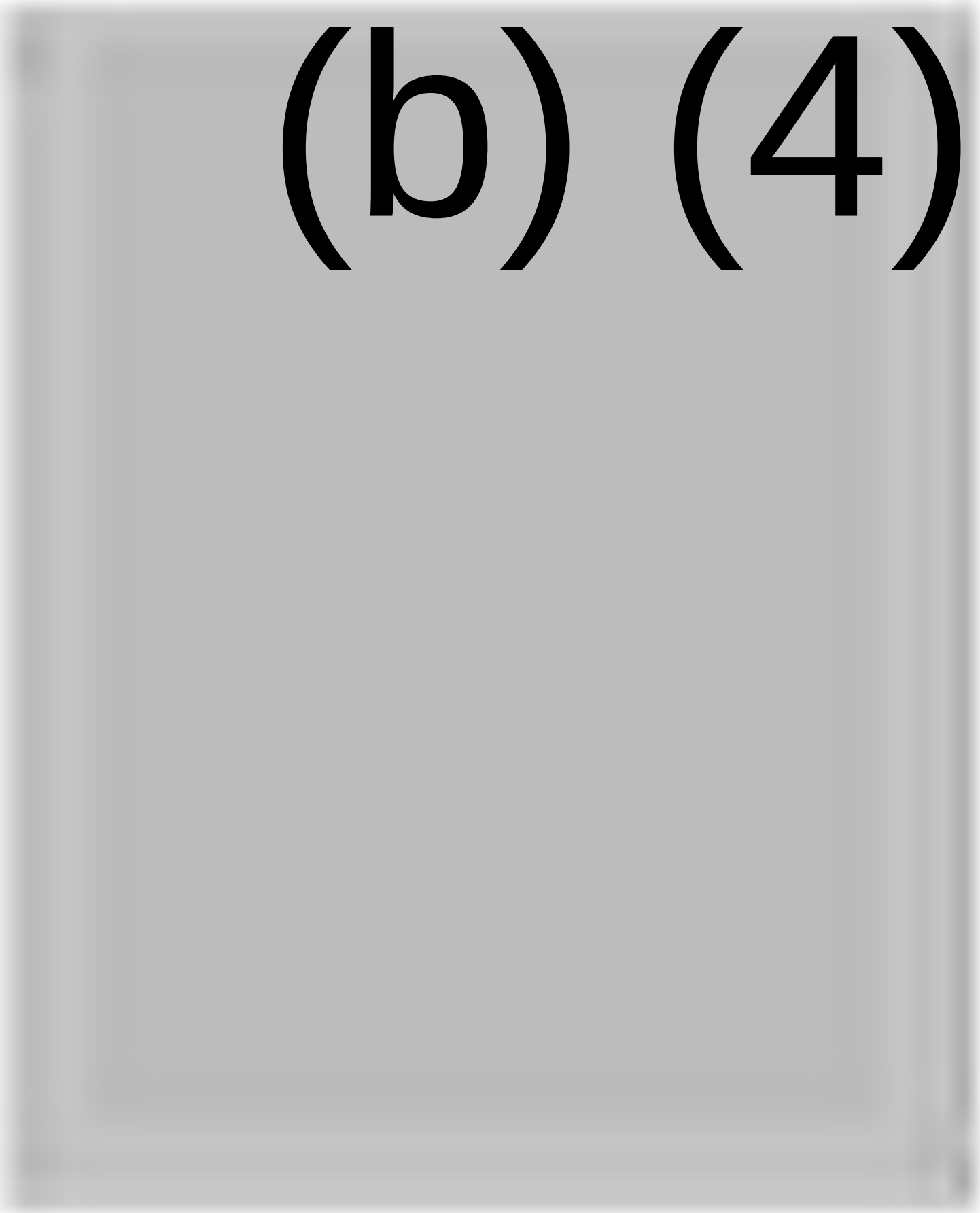
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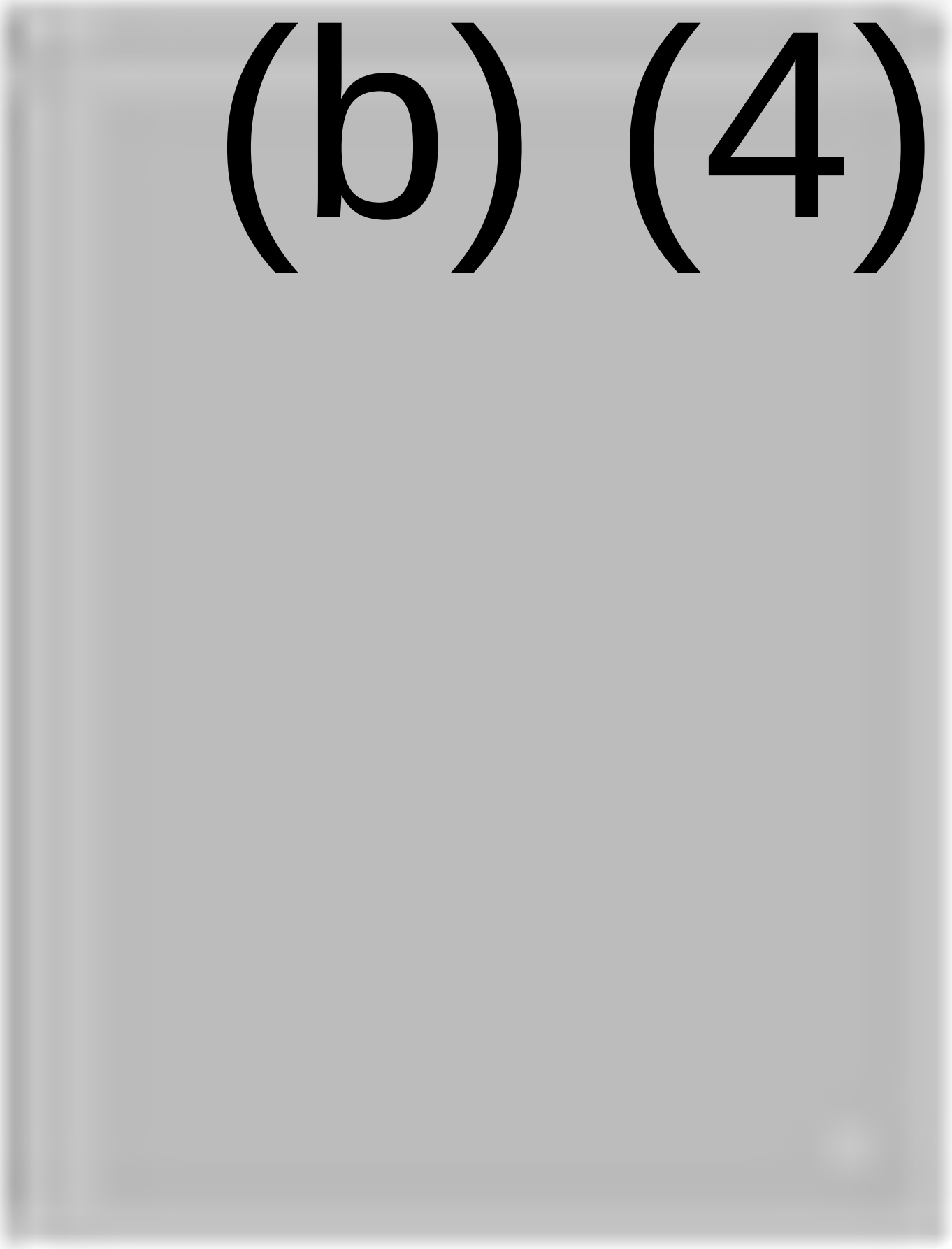
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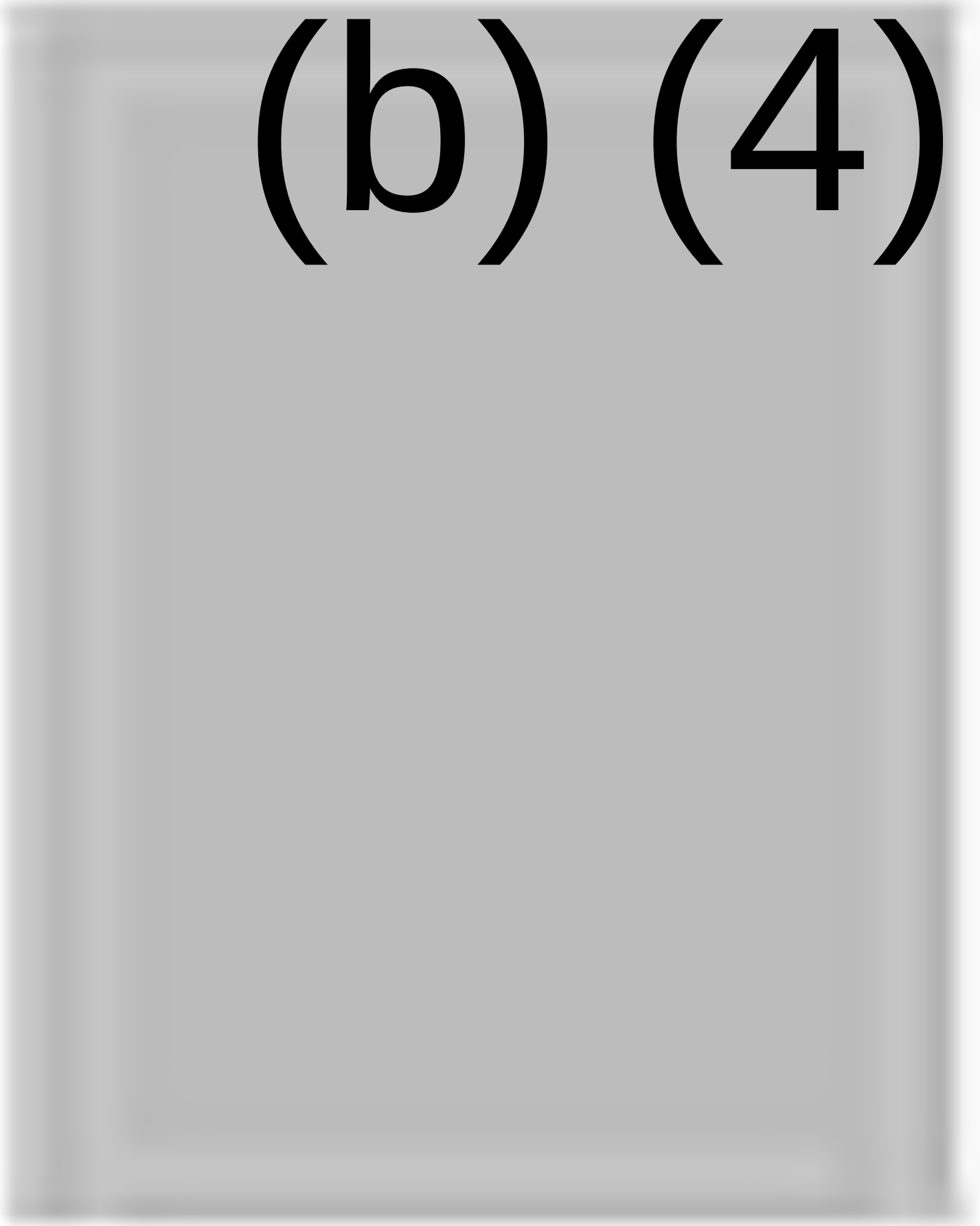
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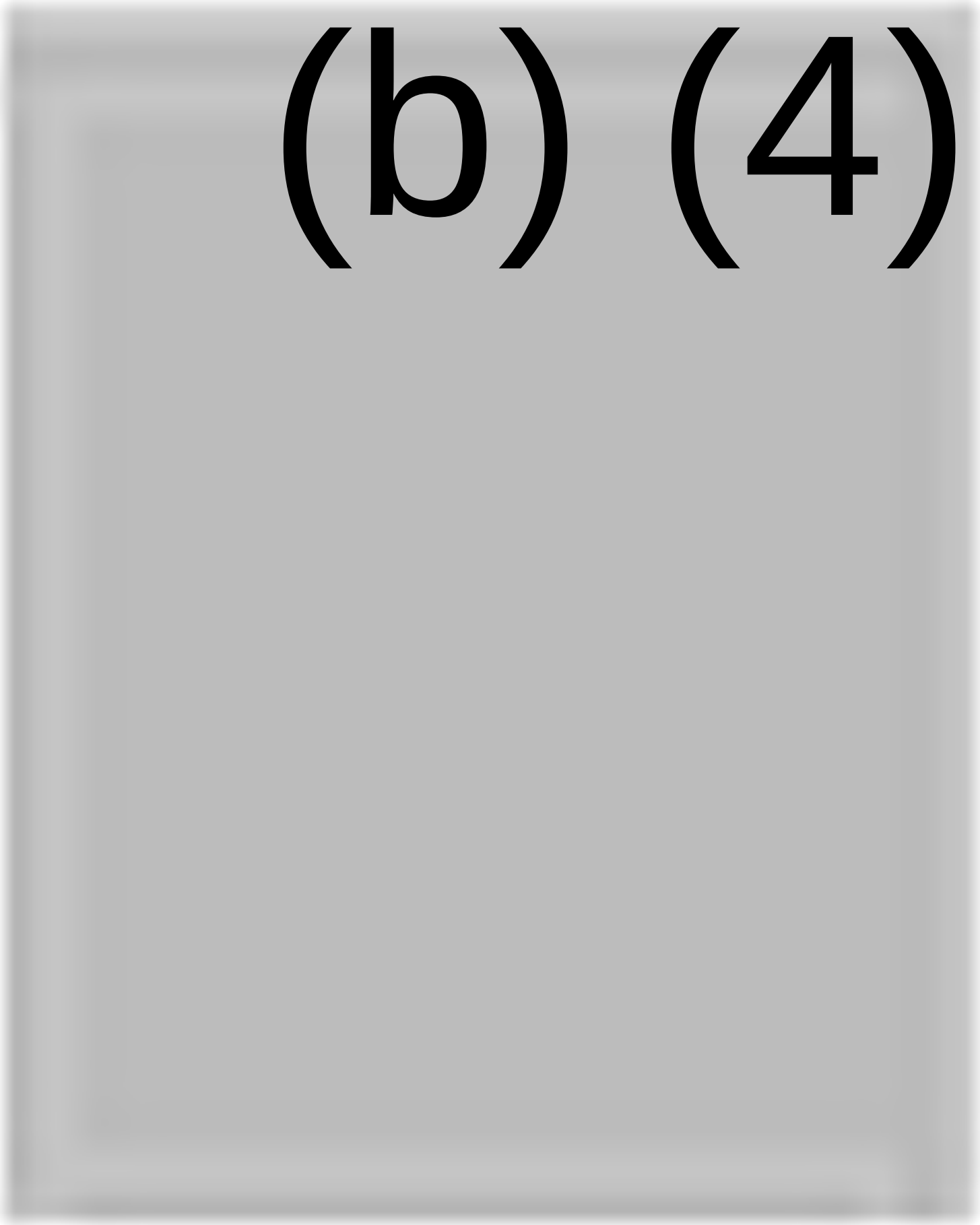
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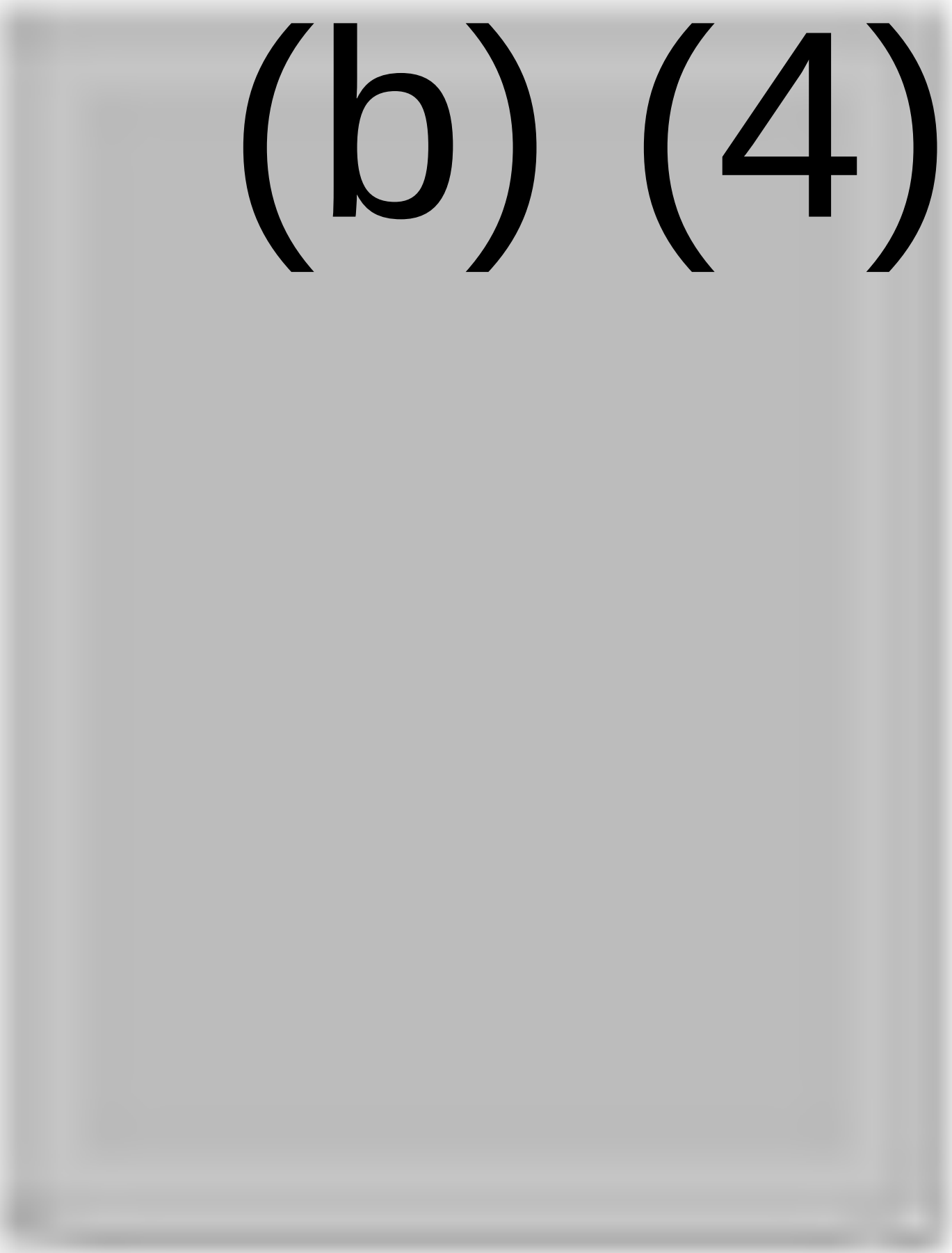
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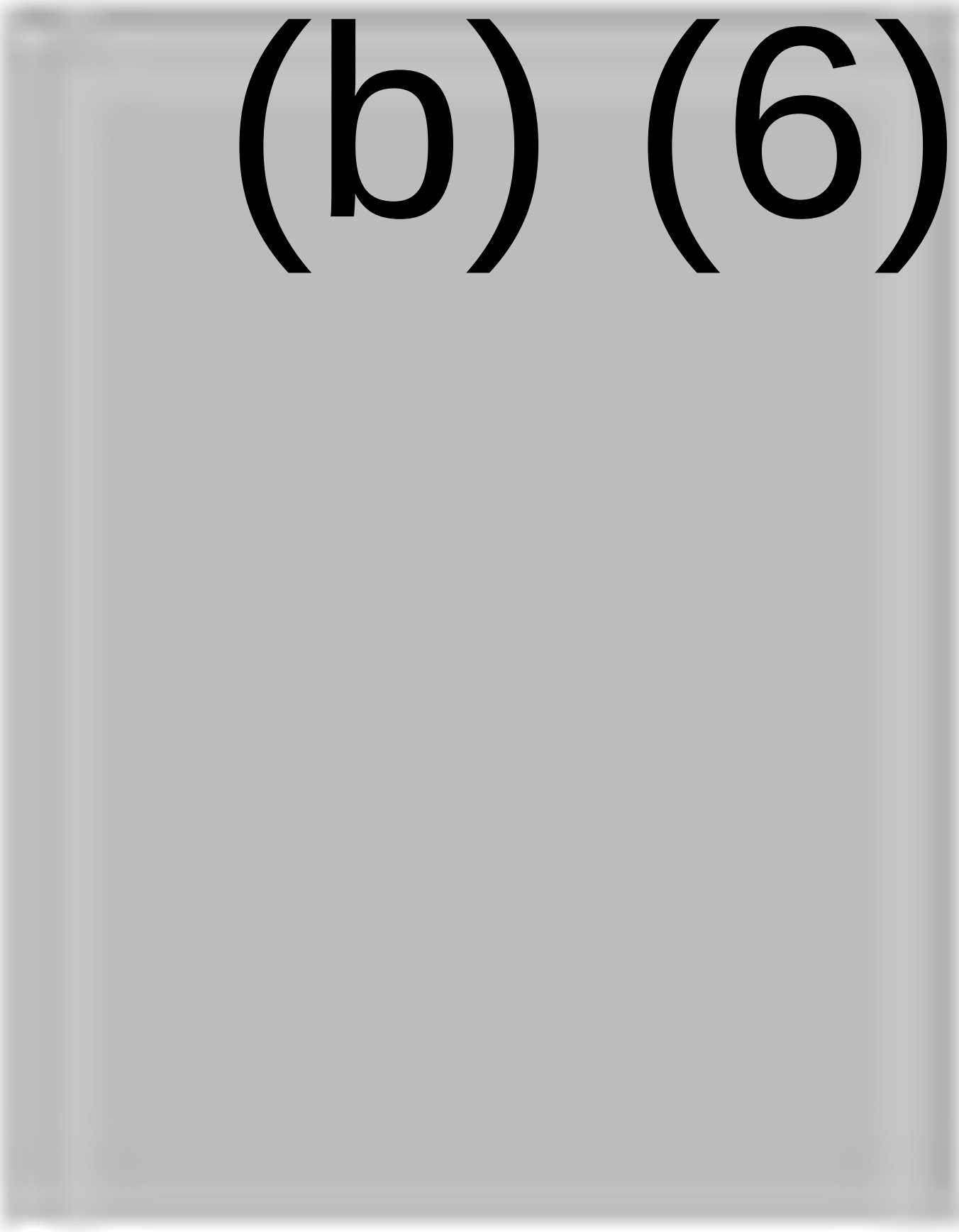
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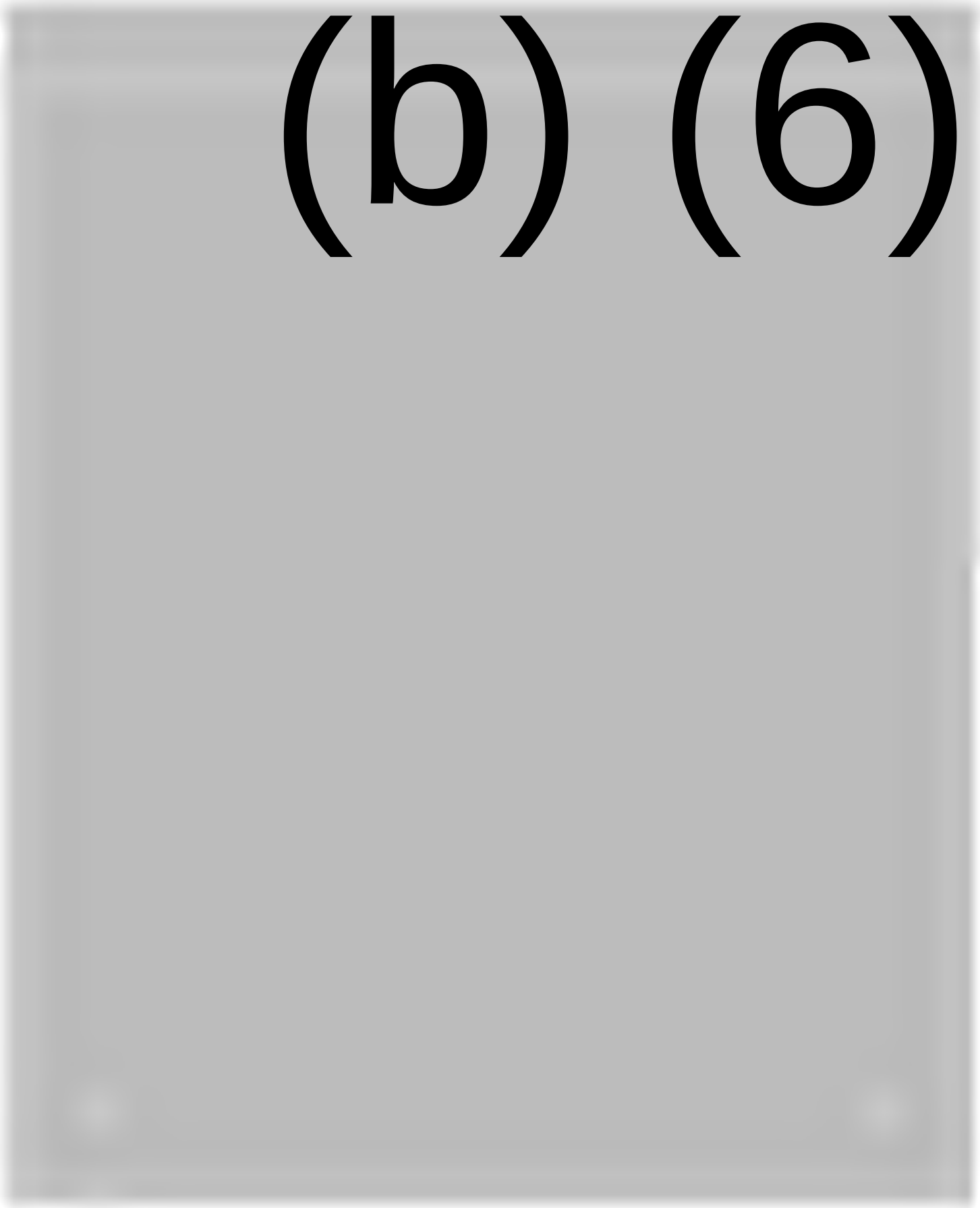
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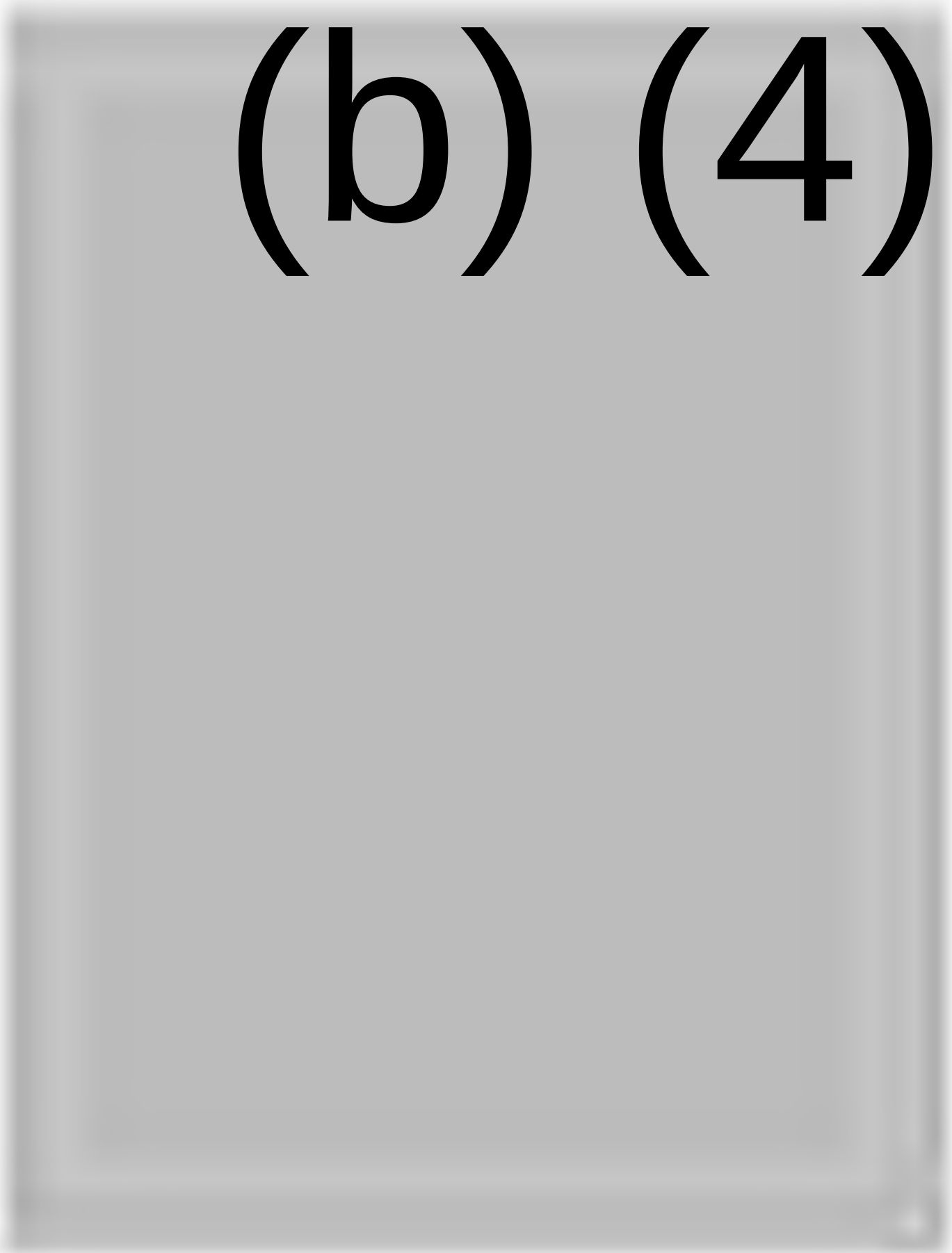
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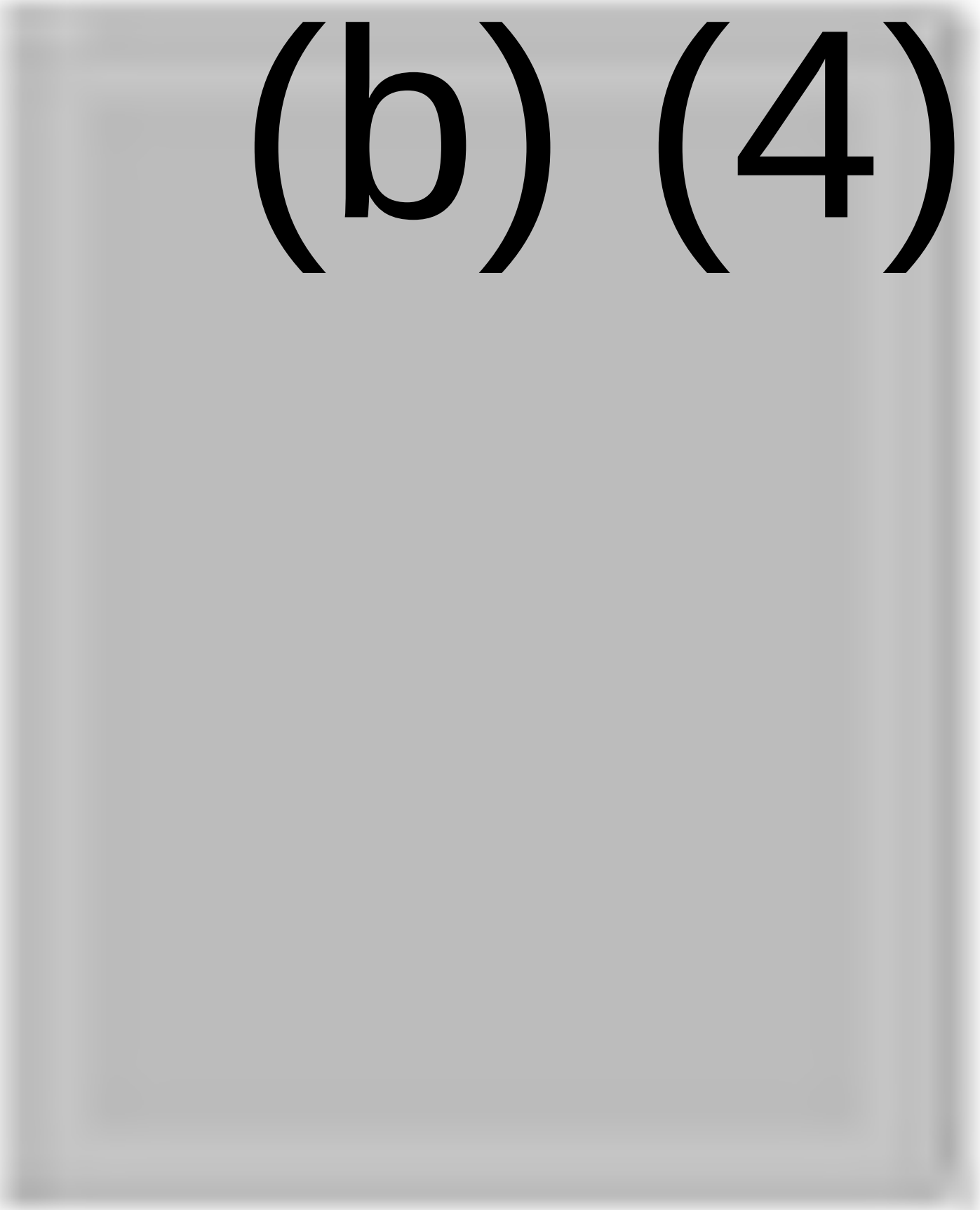
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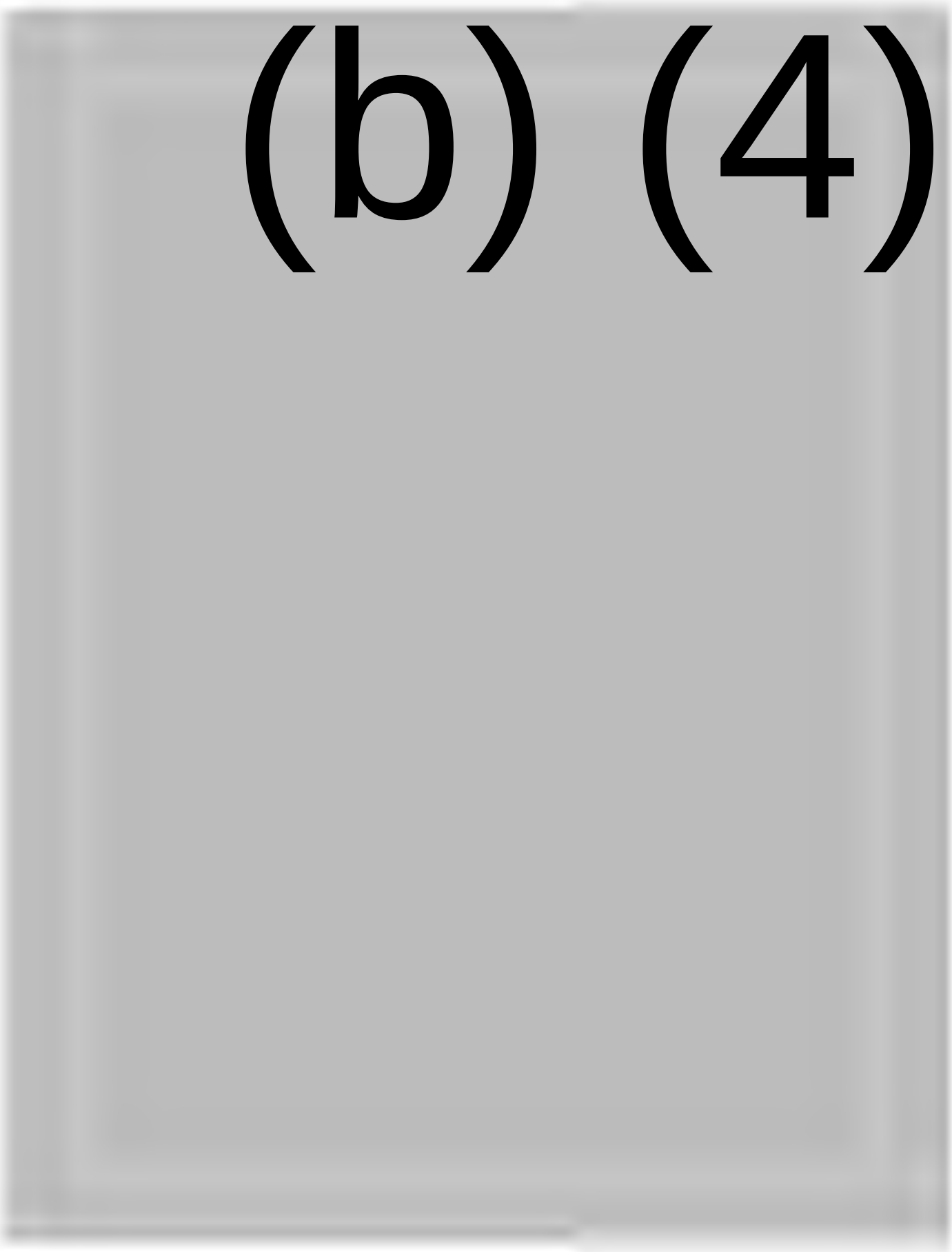
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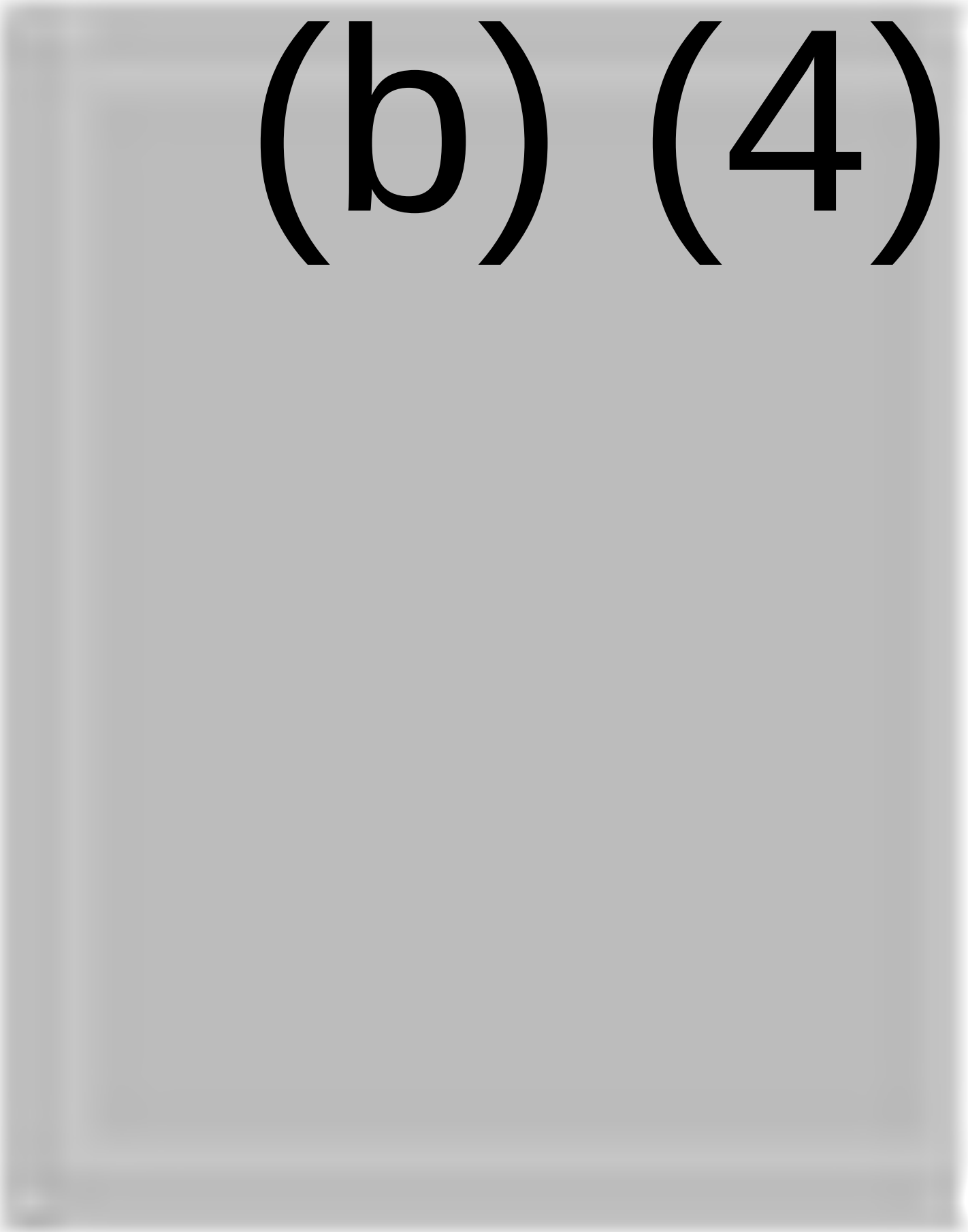
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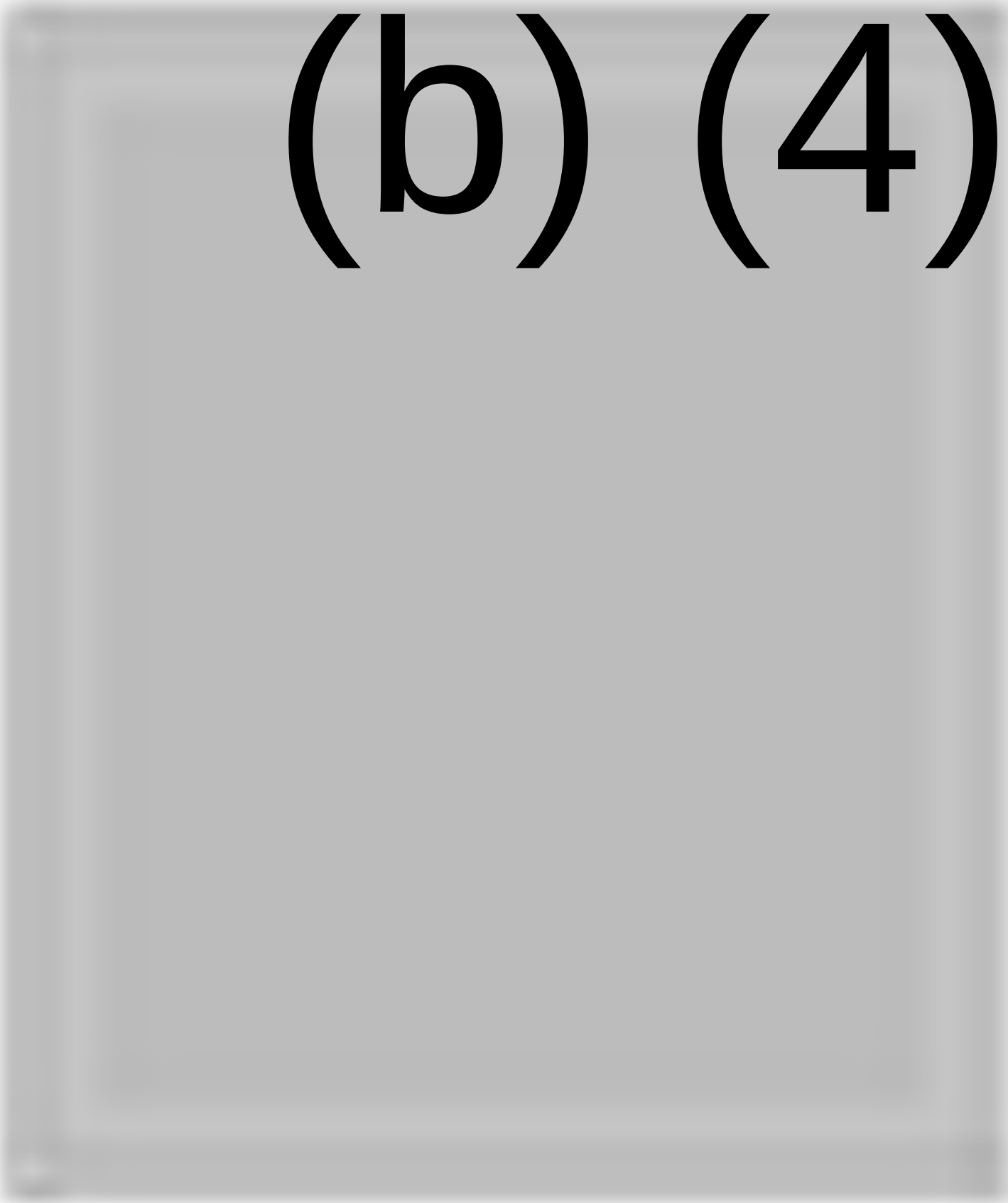
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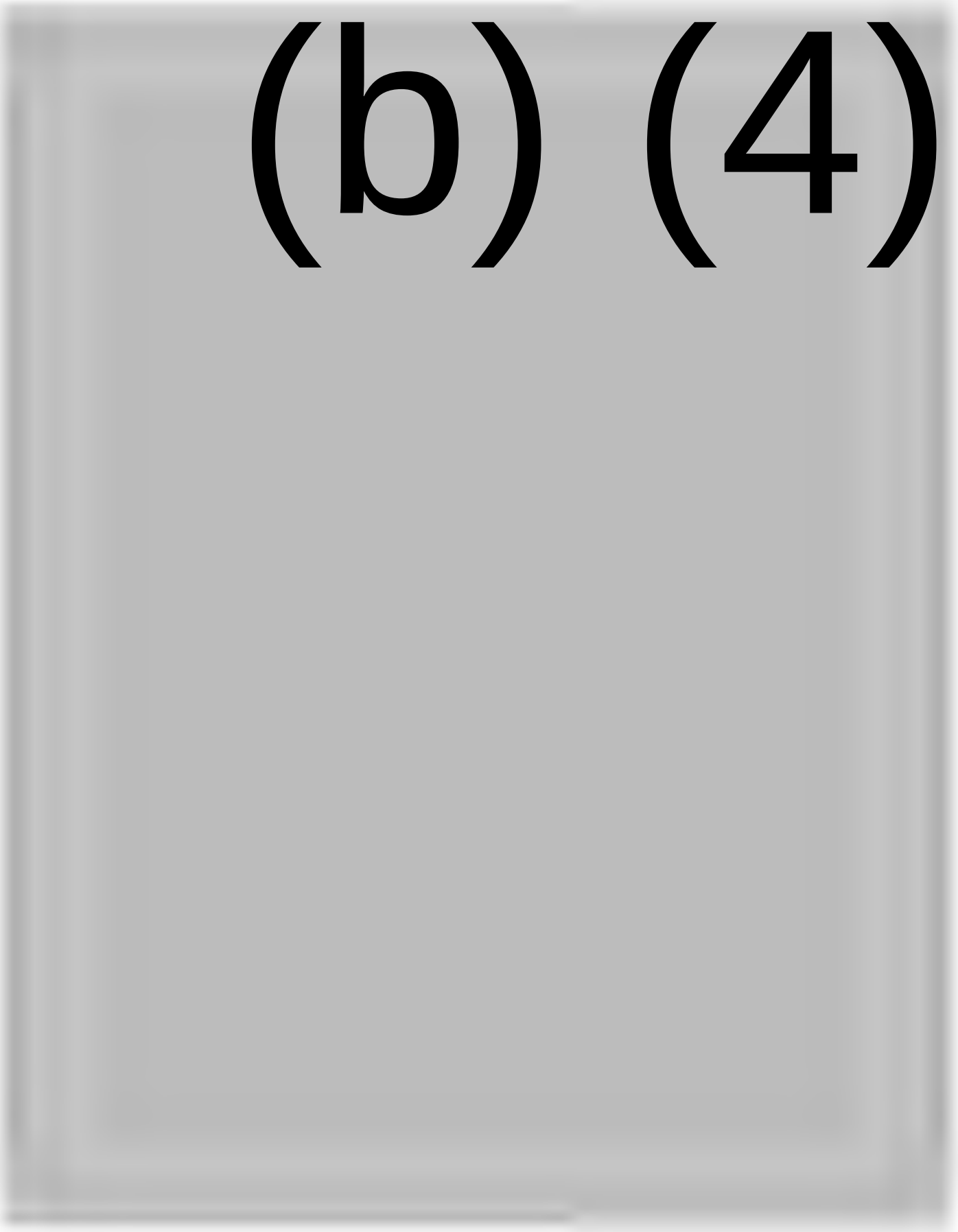
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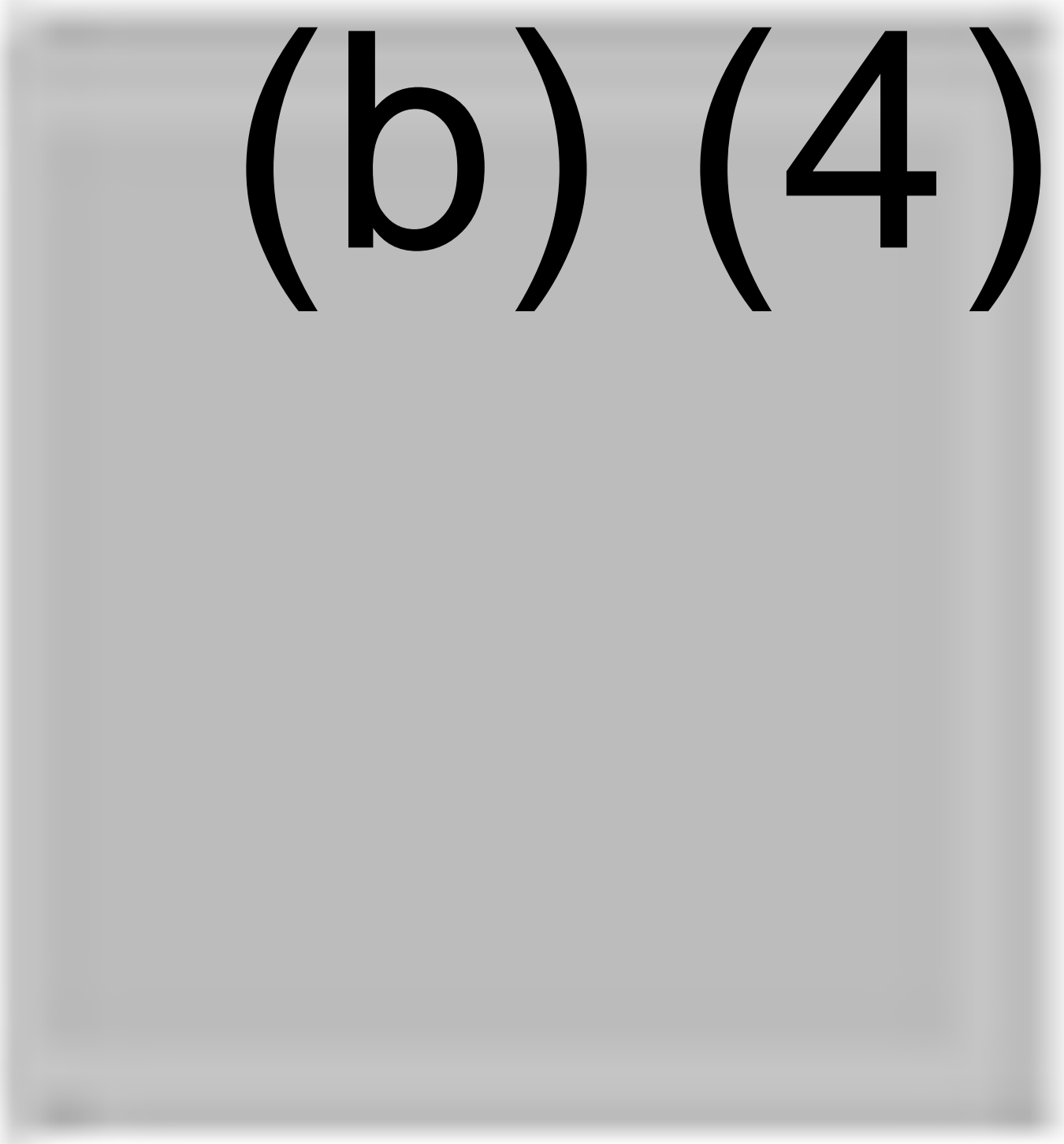

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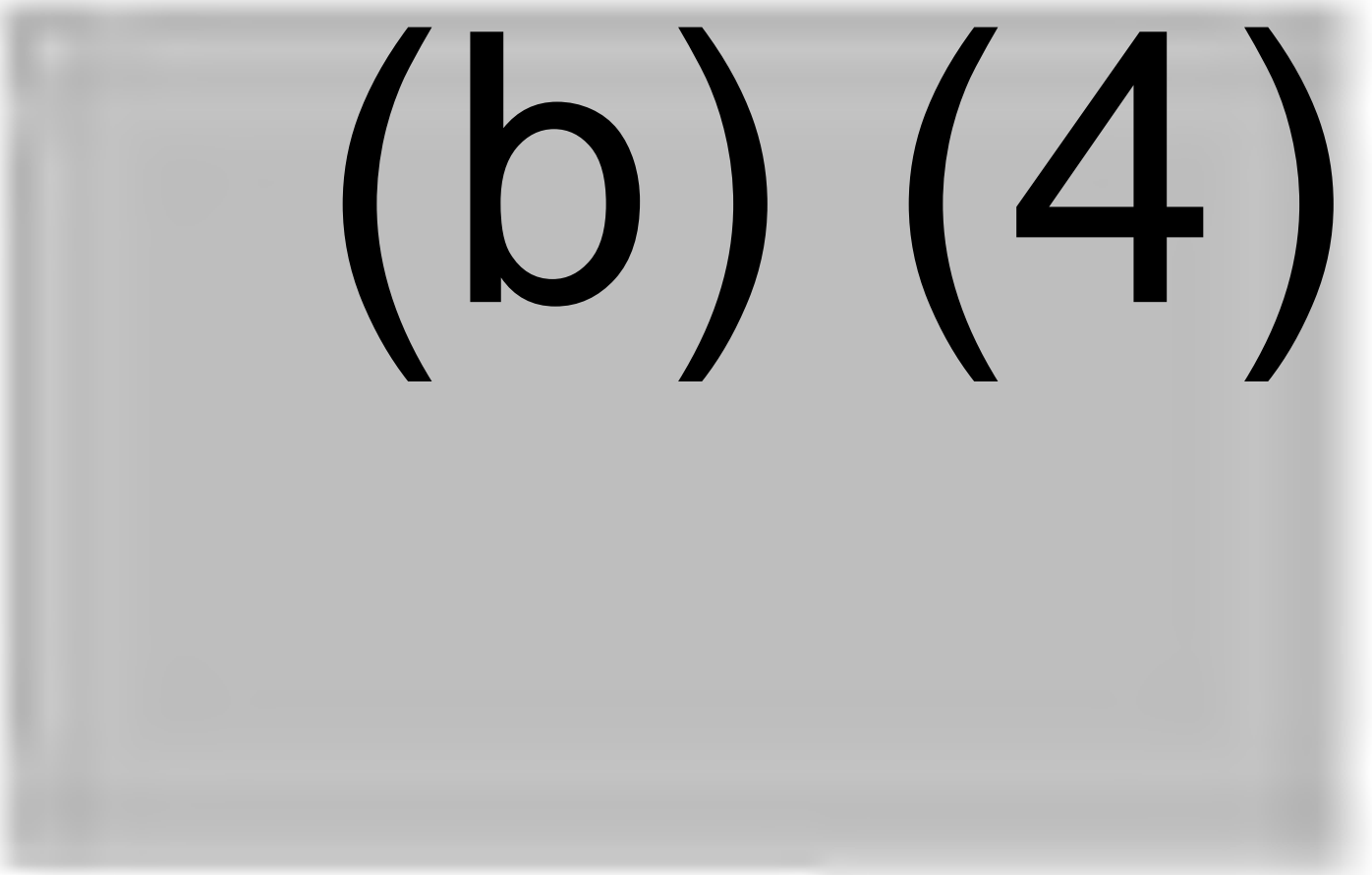
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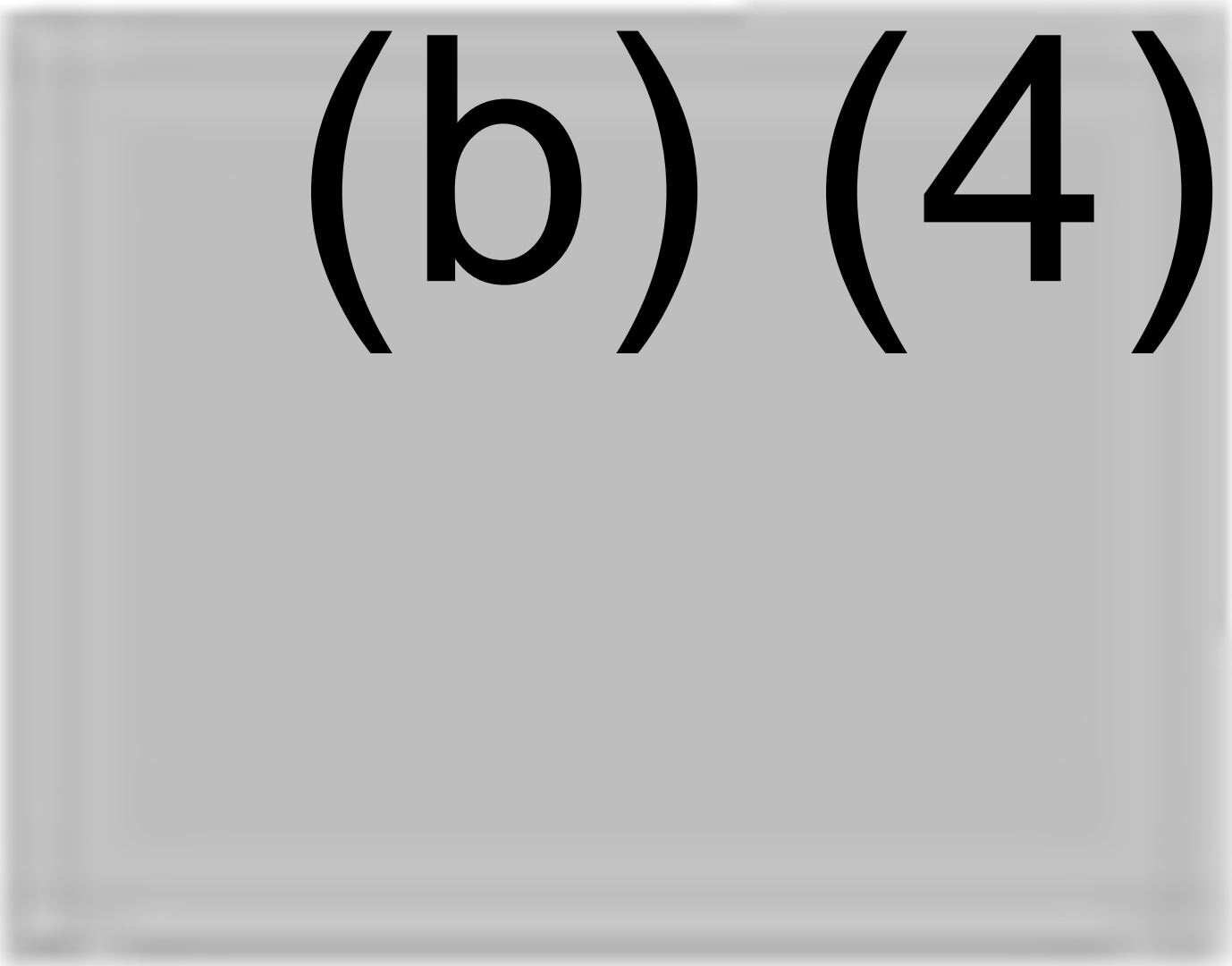
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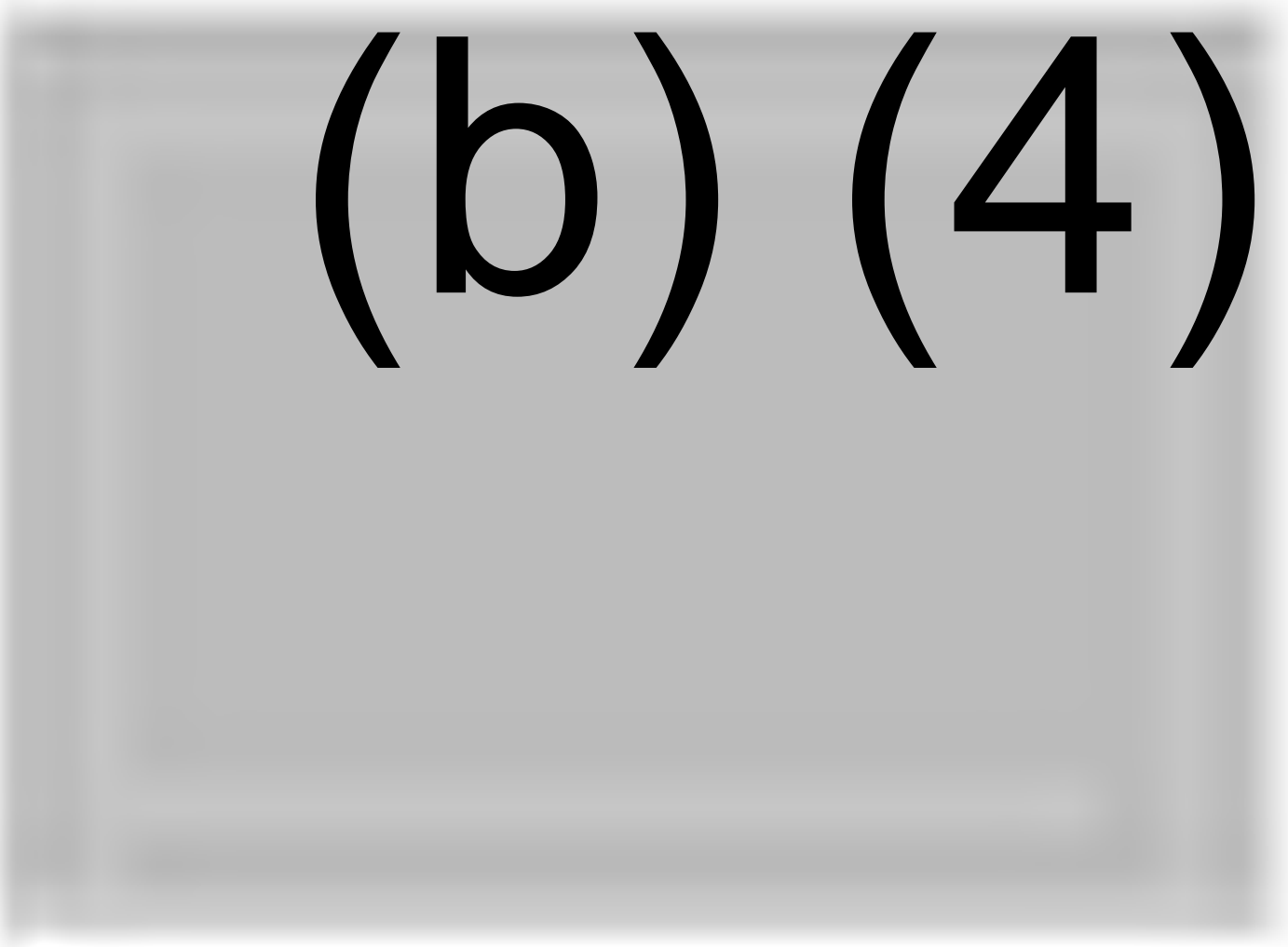
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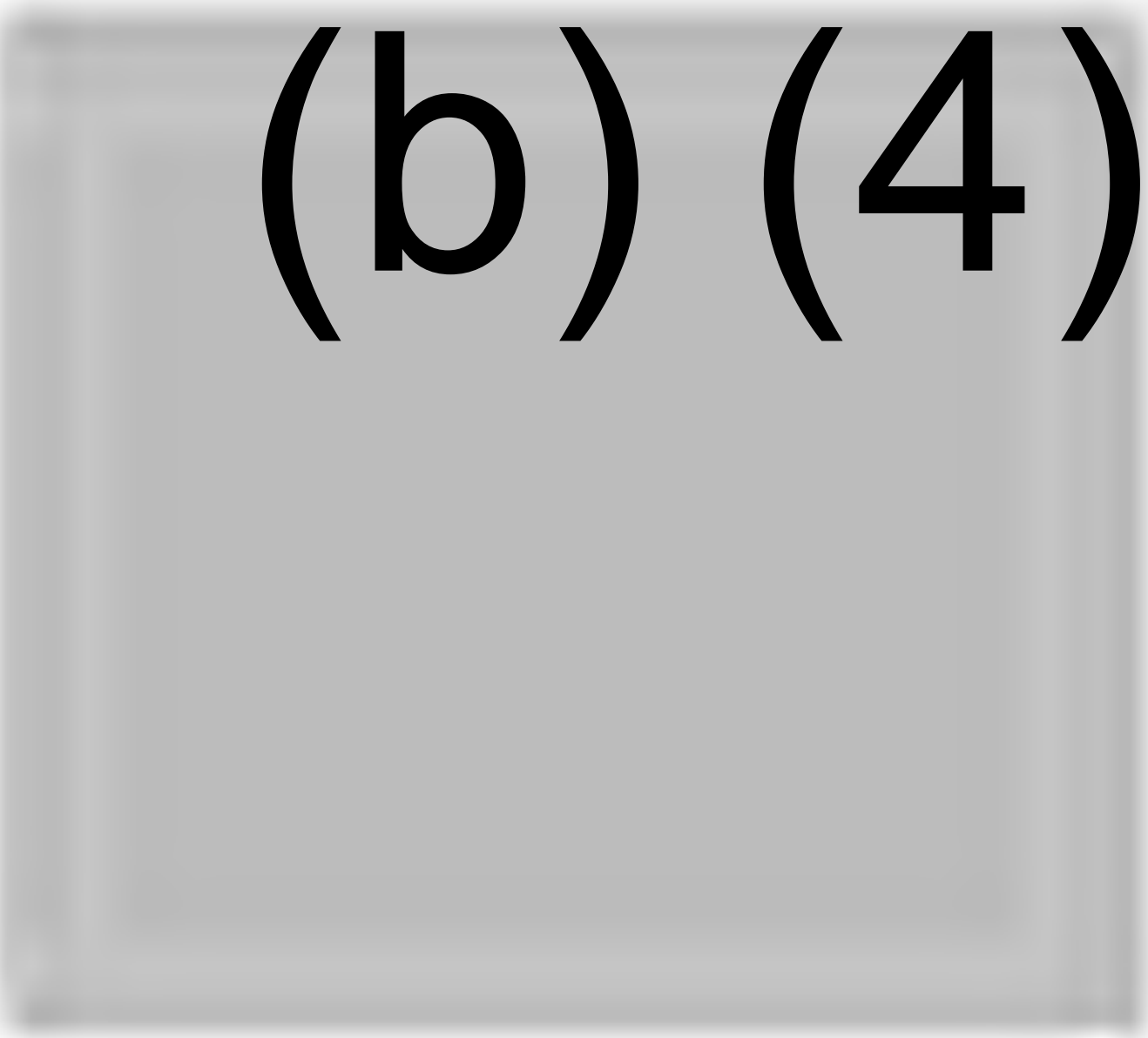
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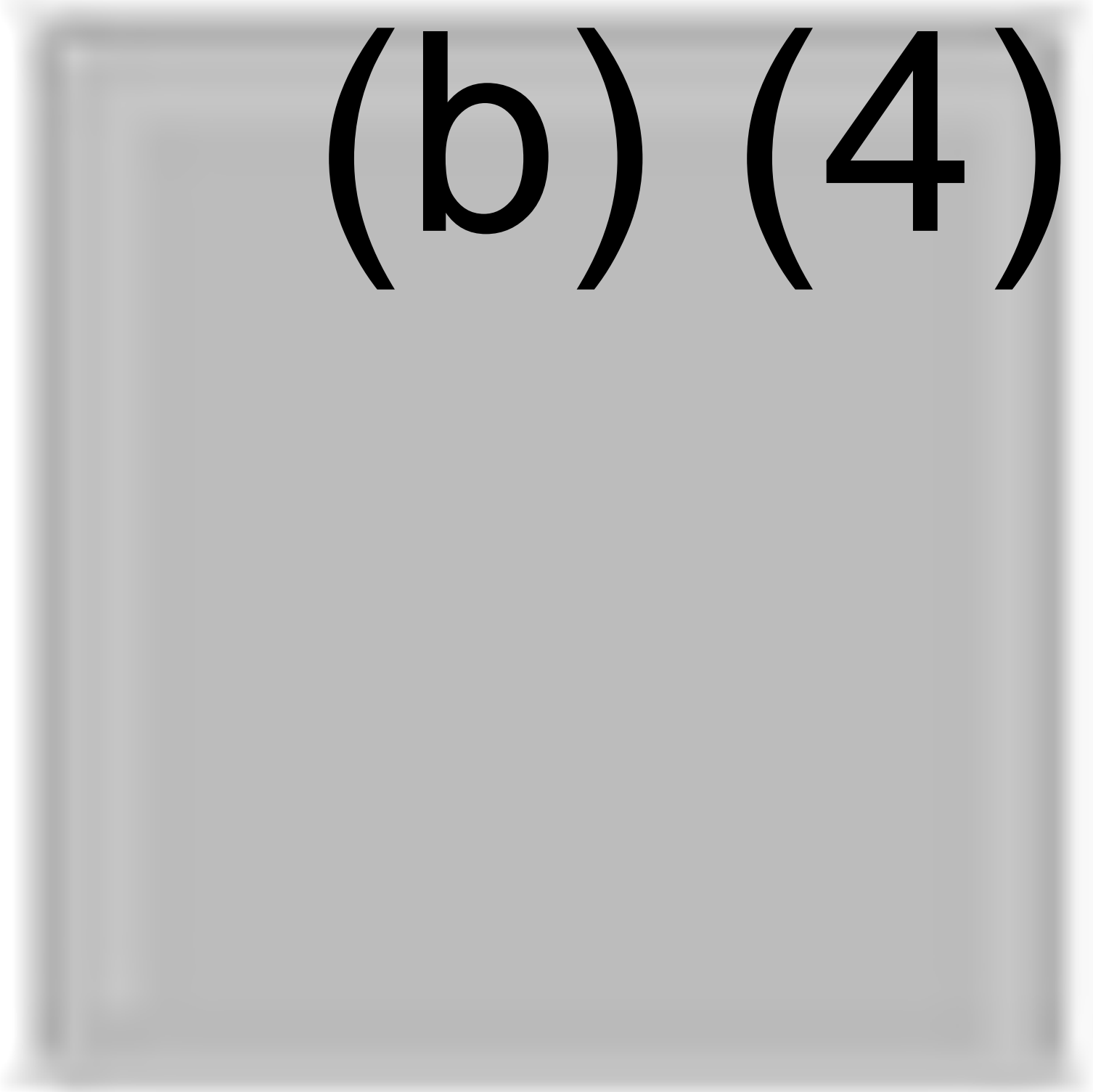

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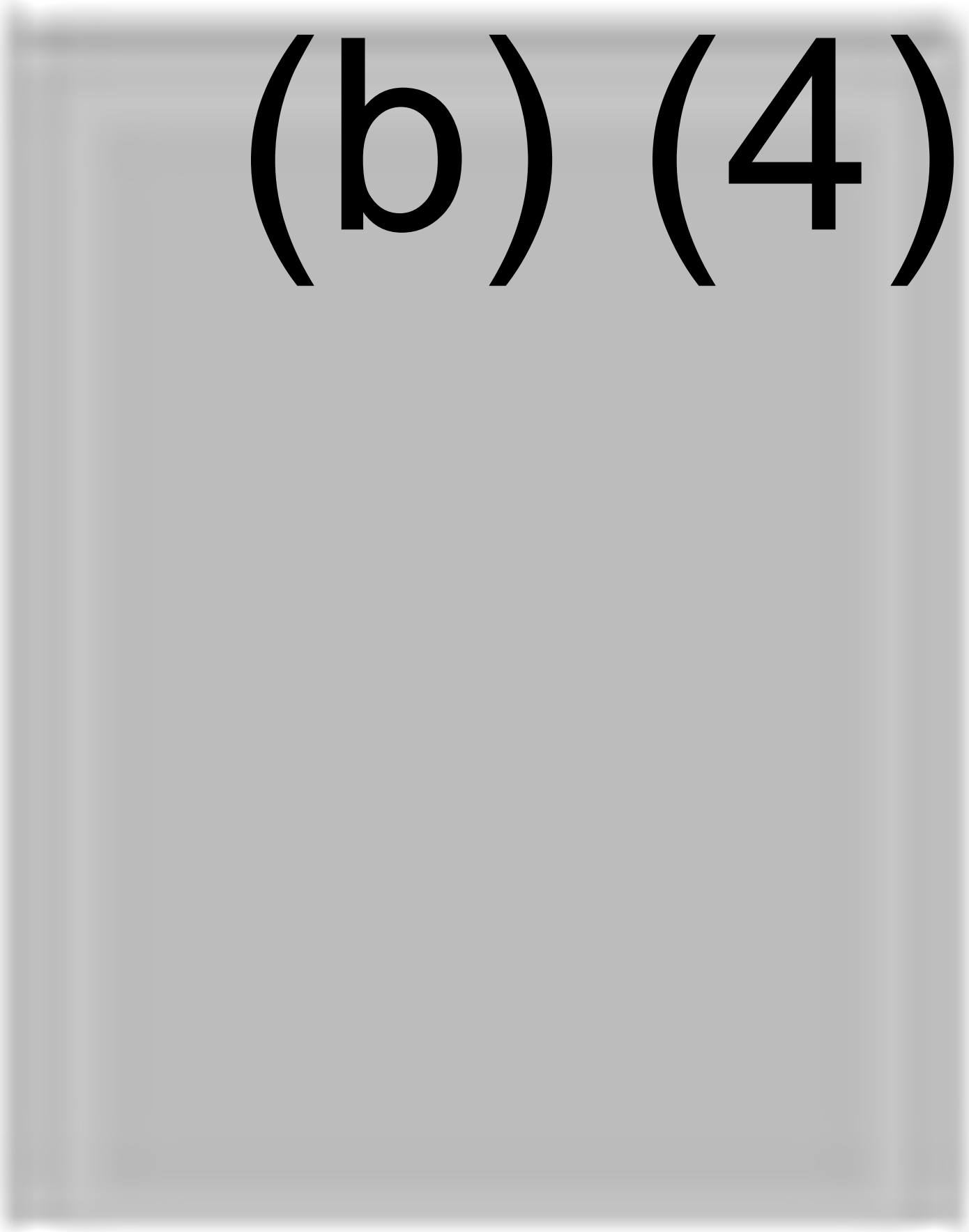
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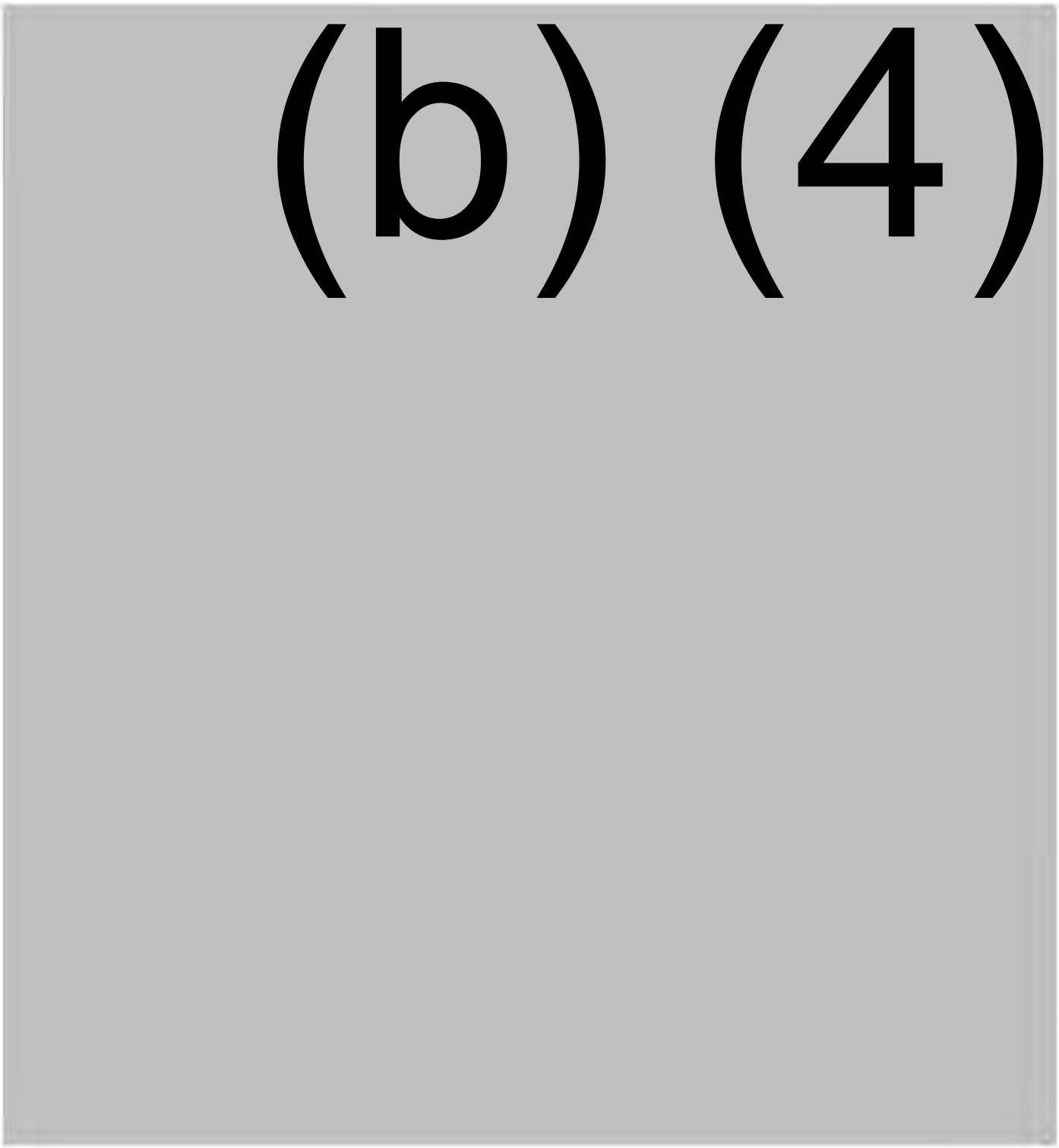
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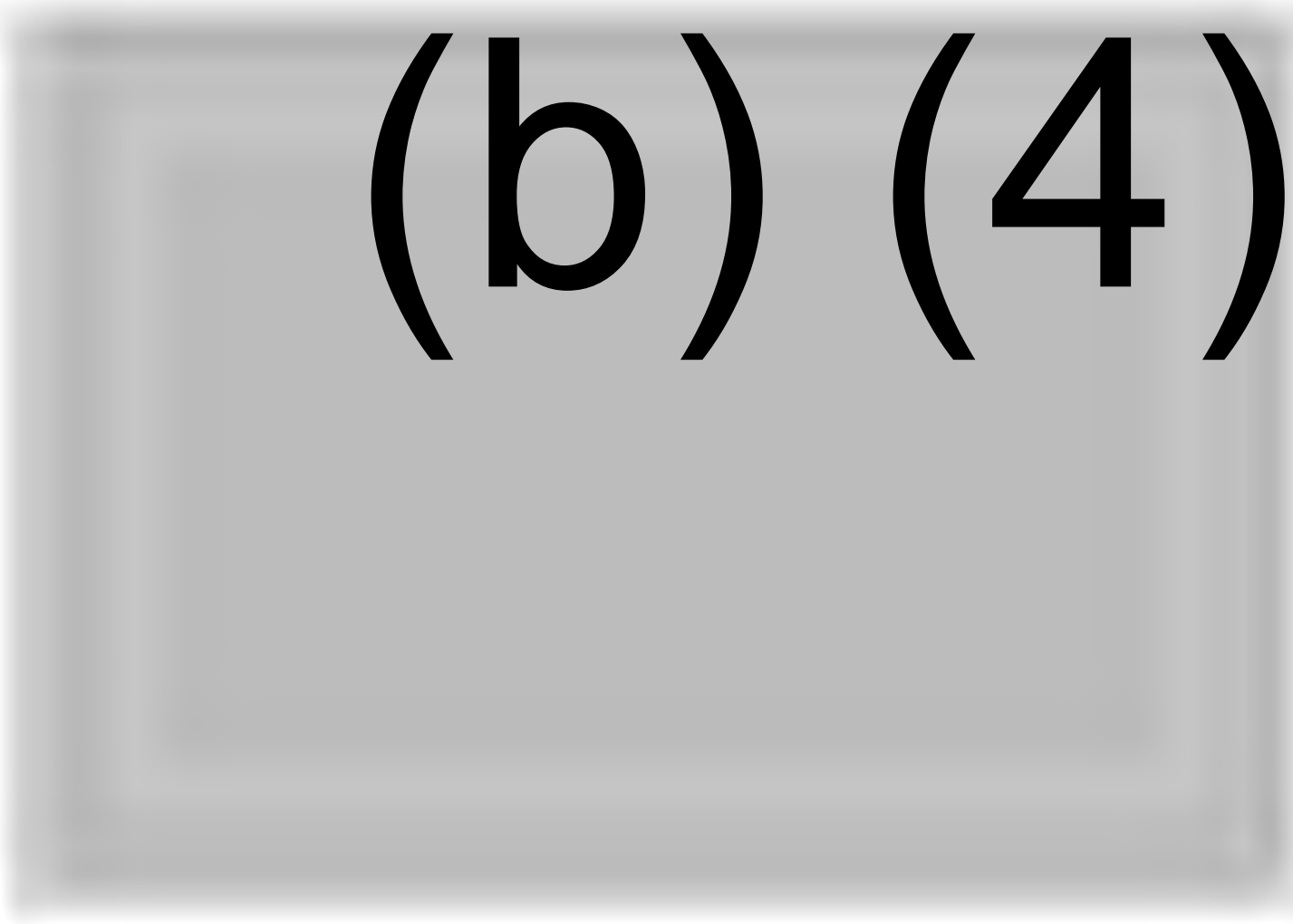
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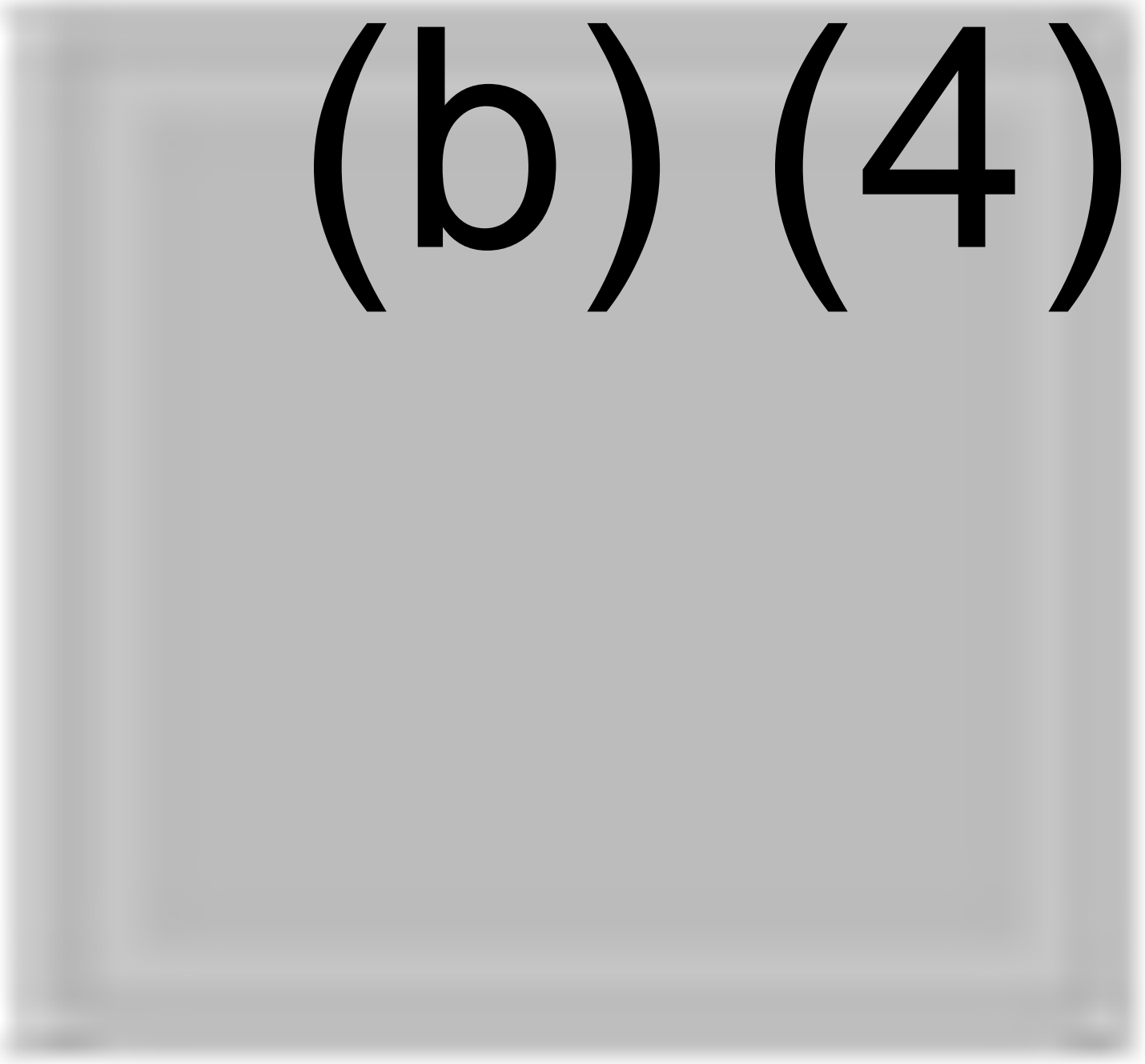
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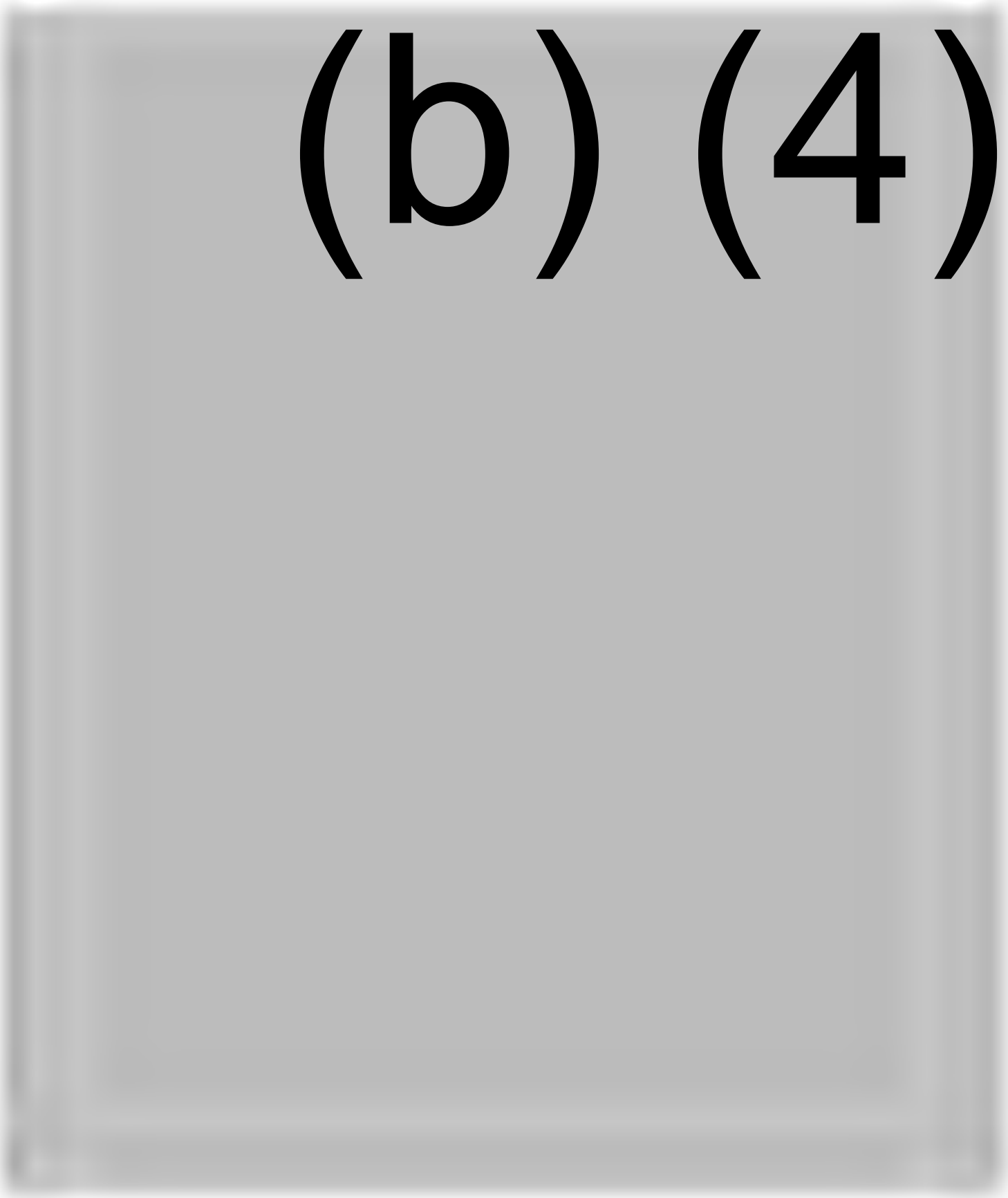
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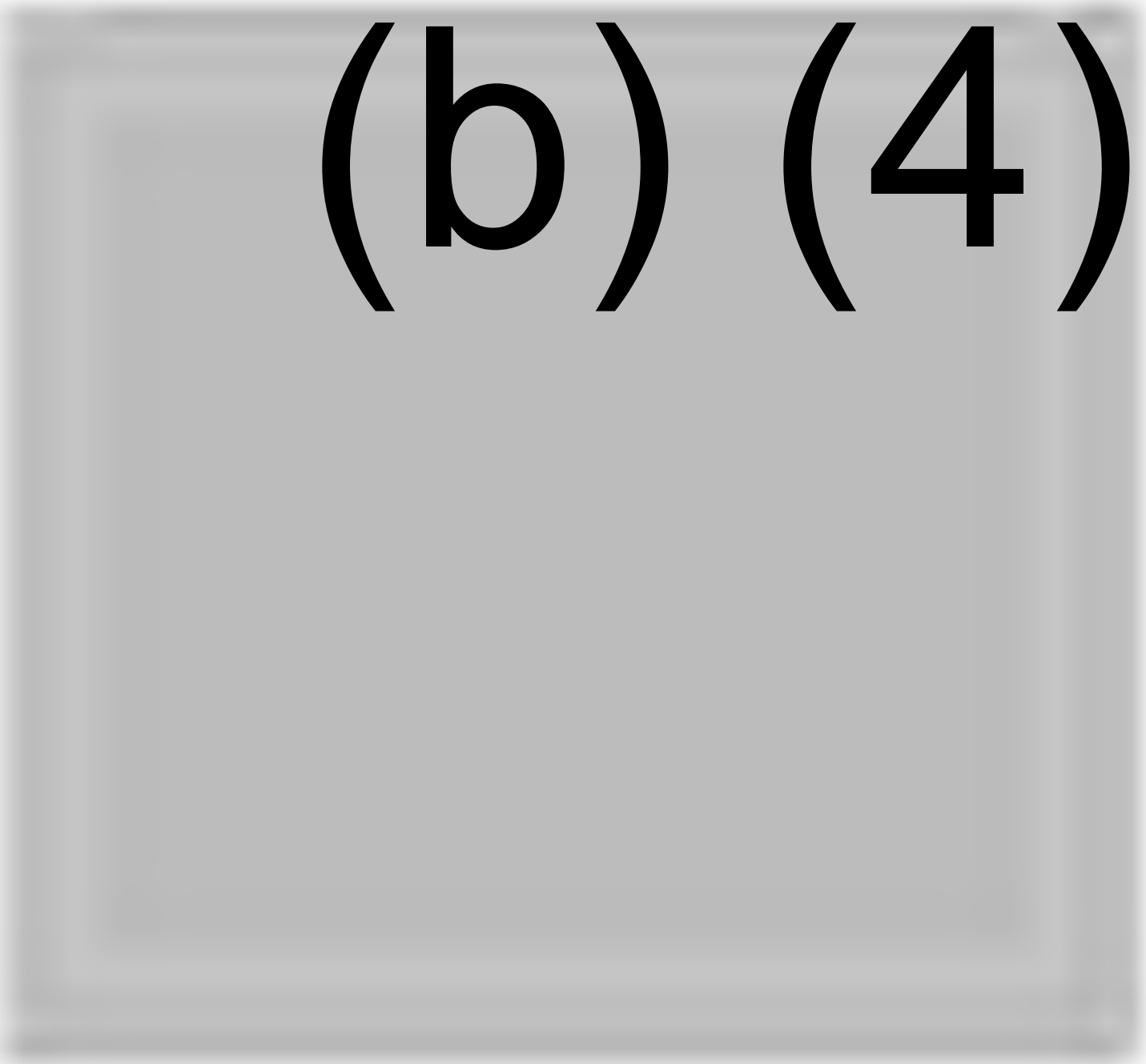
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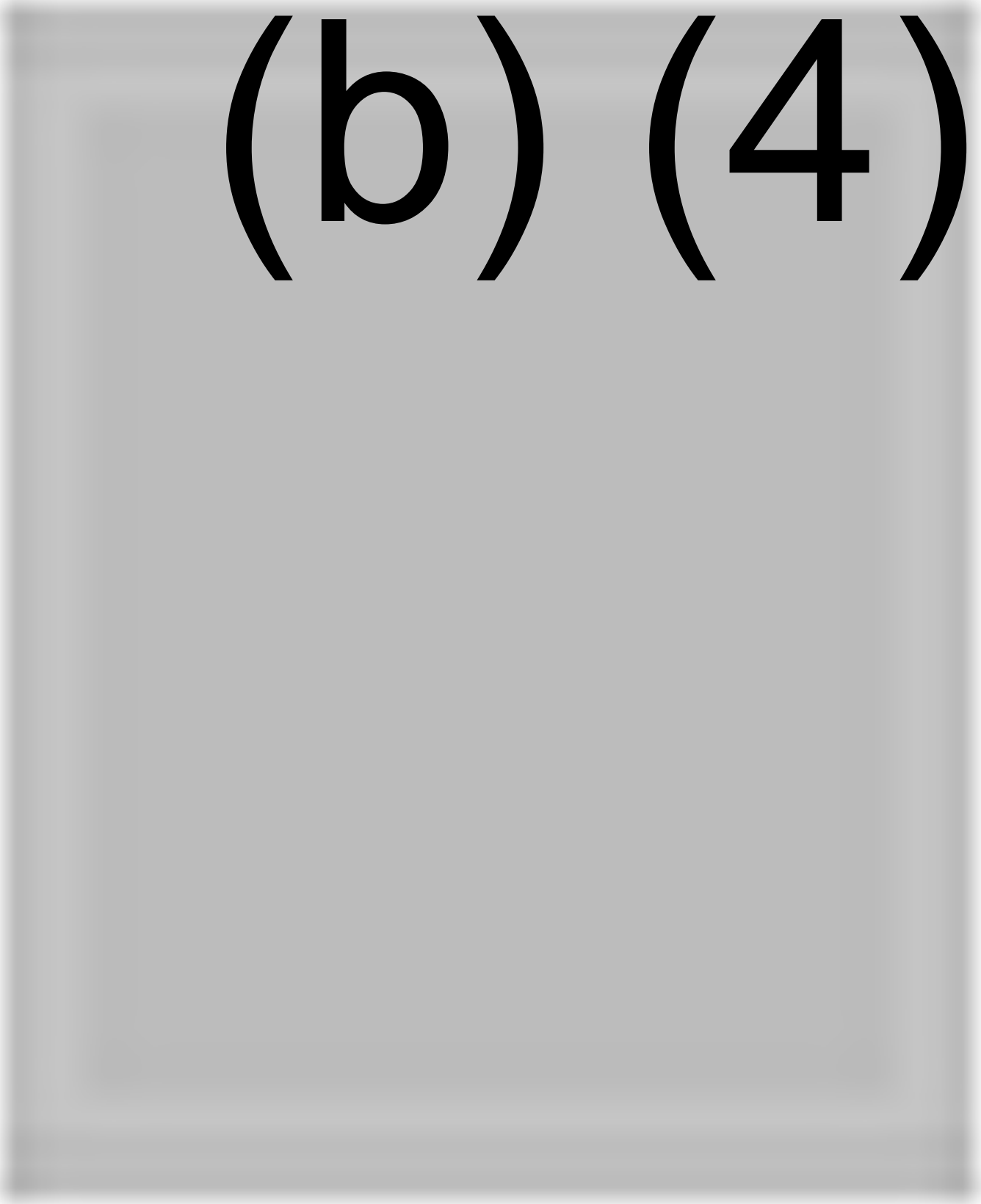
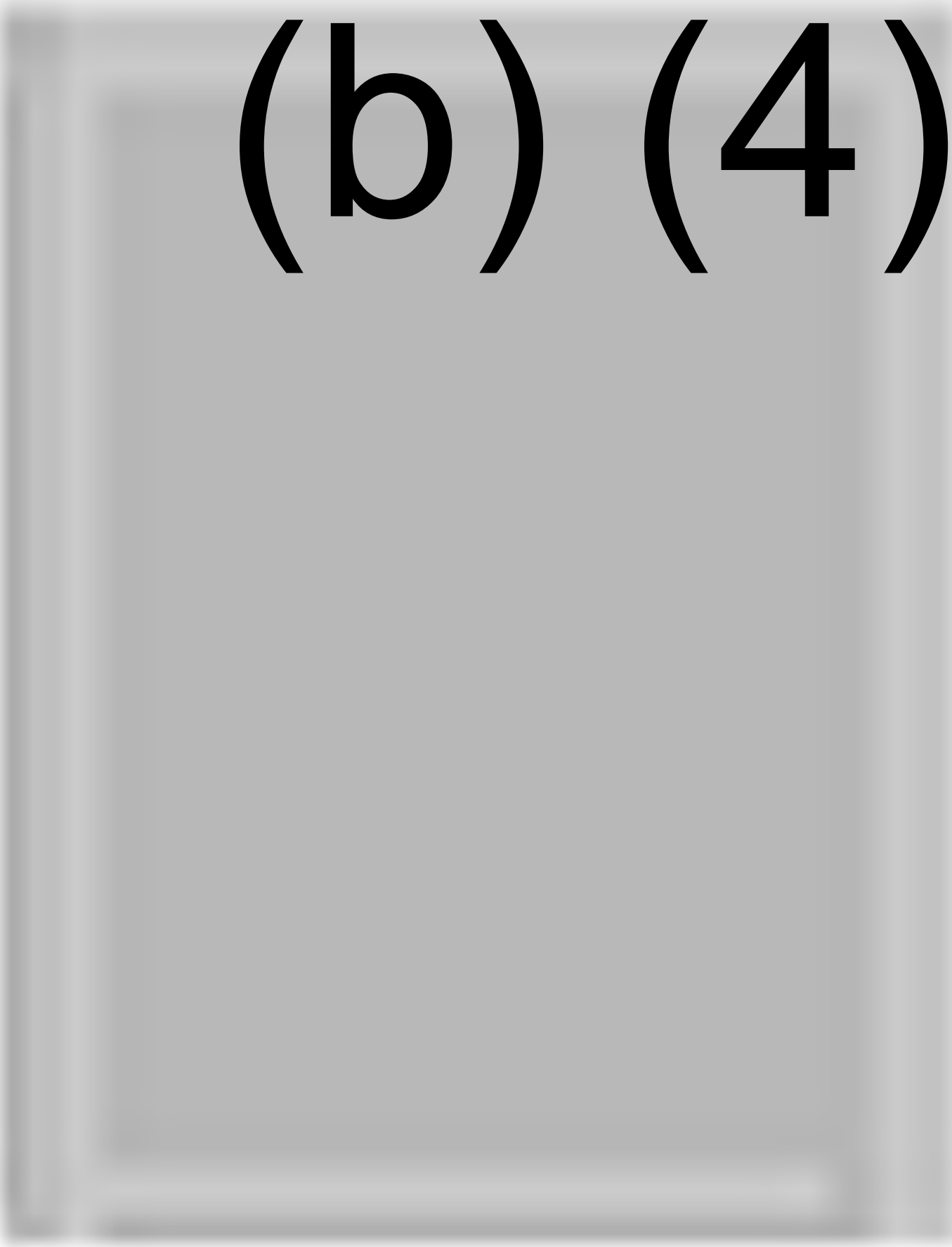
943 ***REFERENCES***

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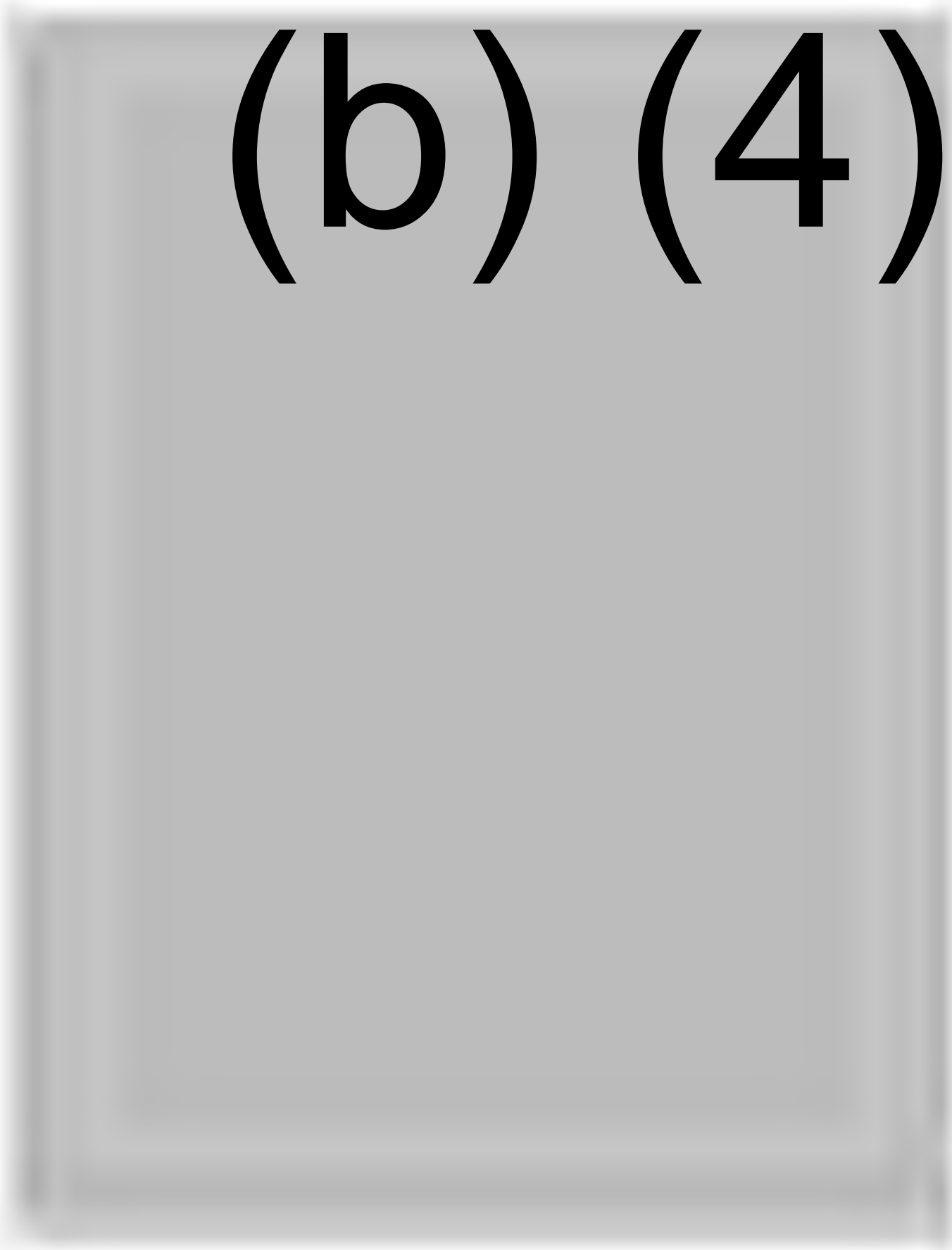
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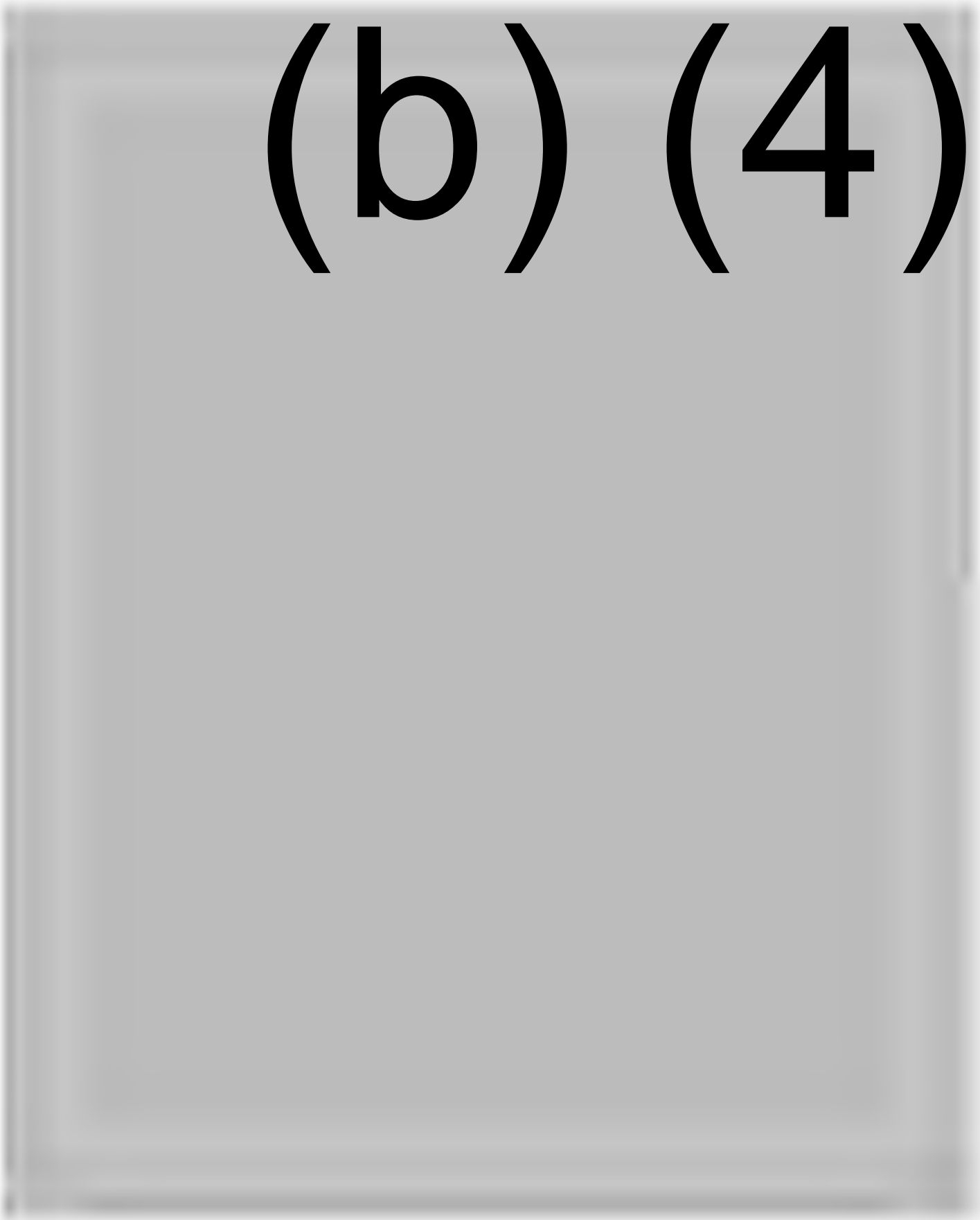
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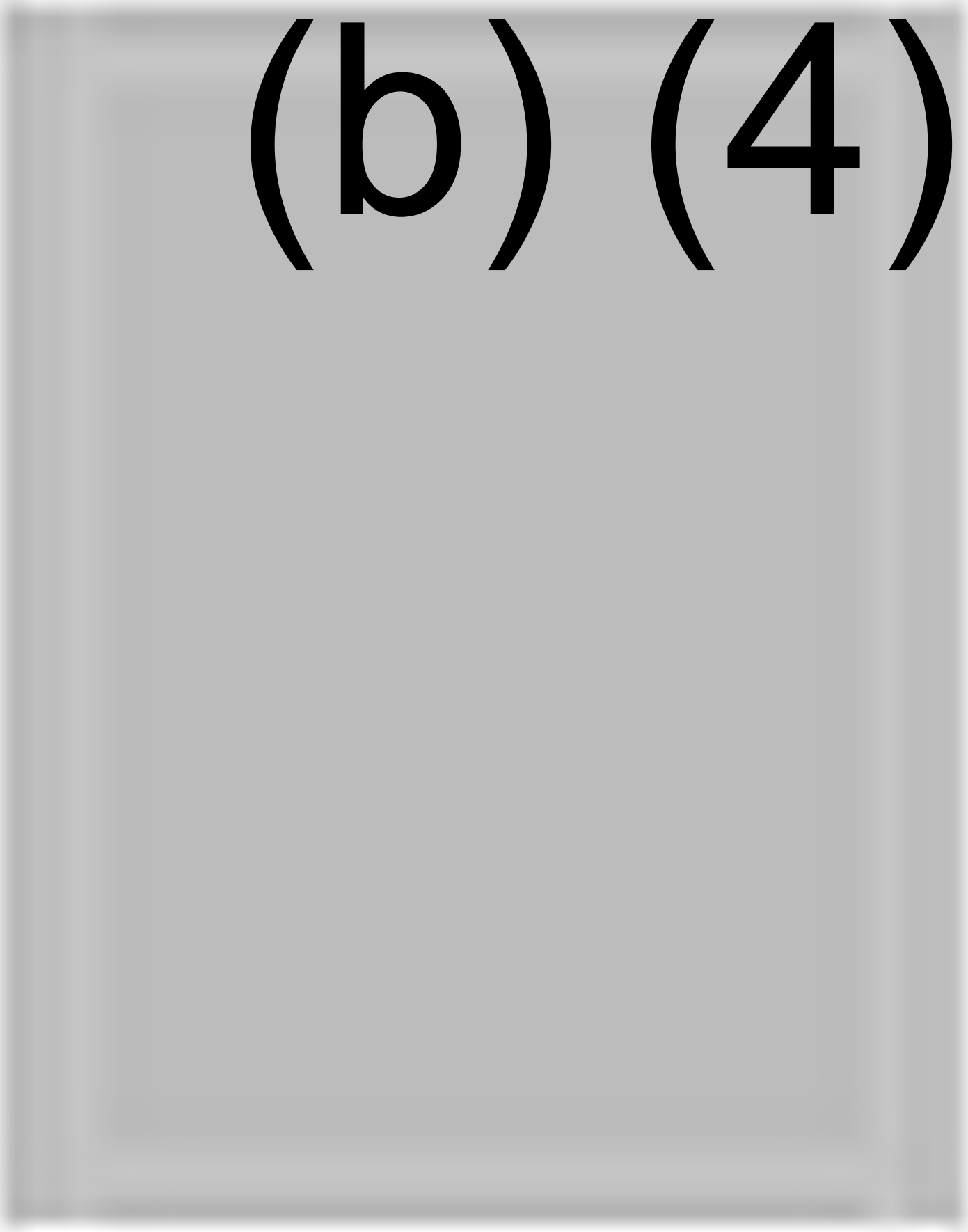
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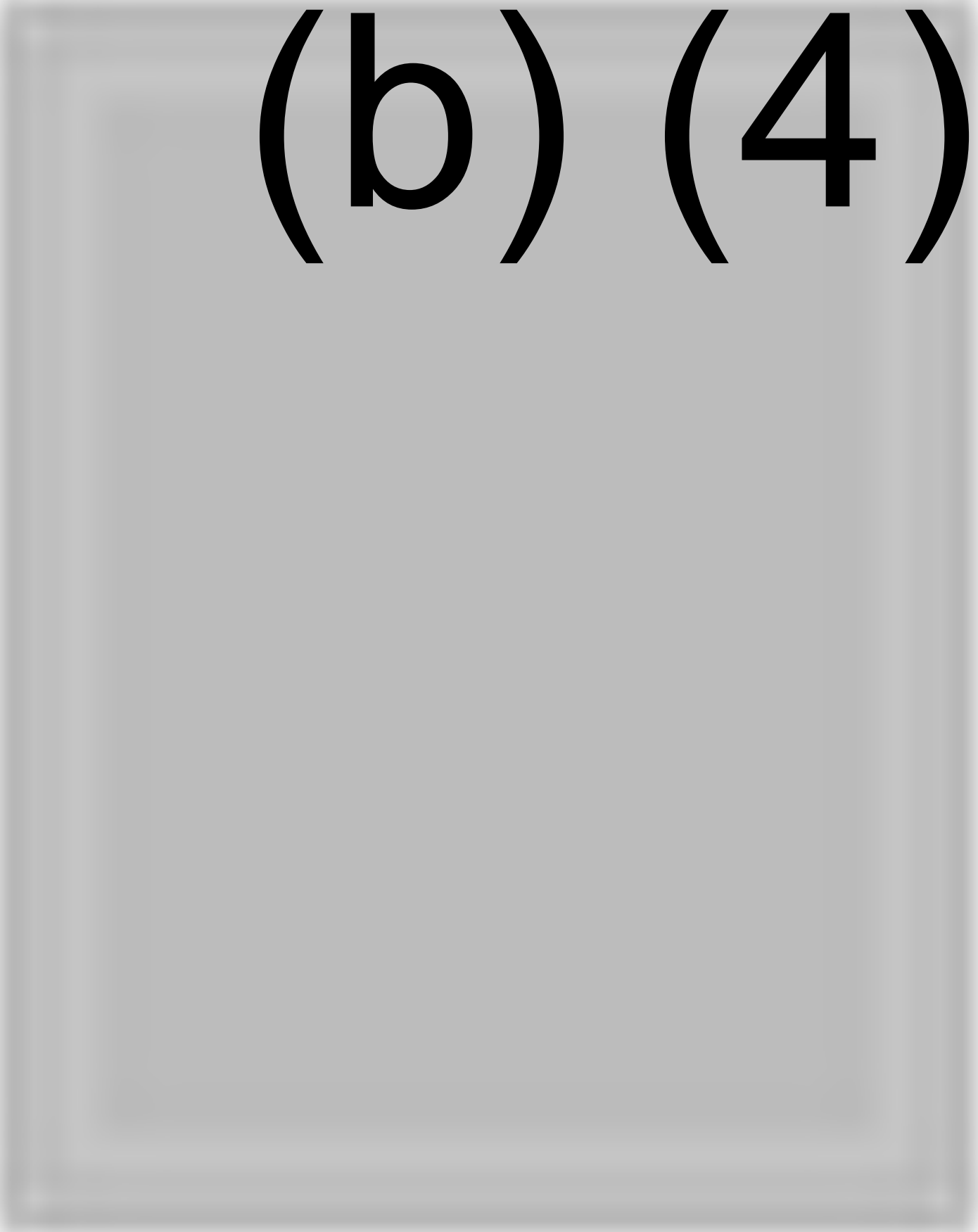
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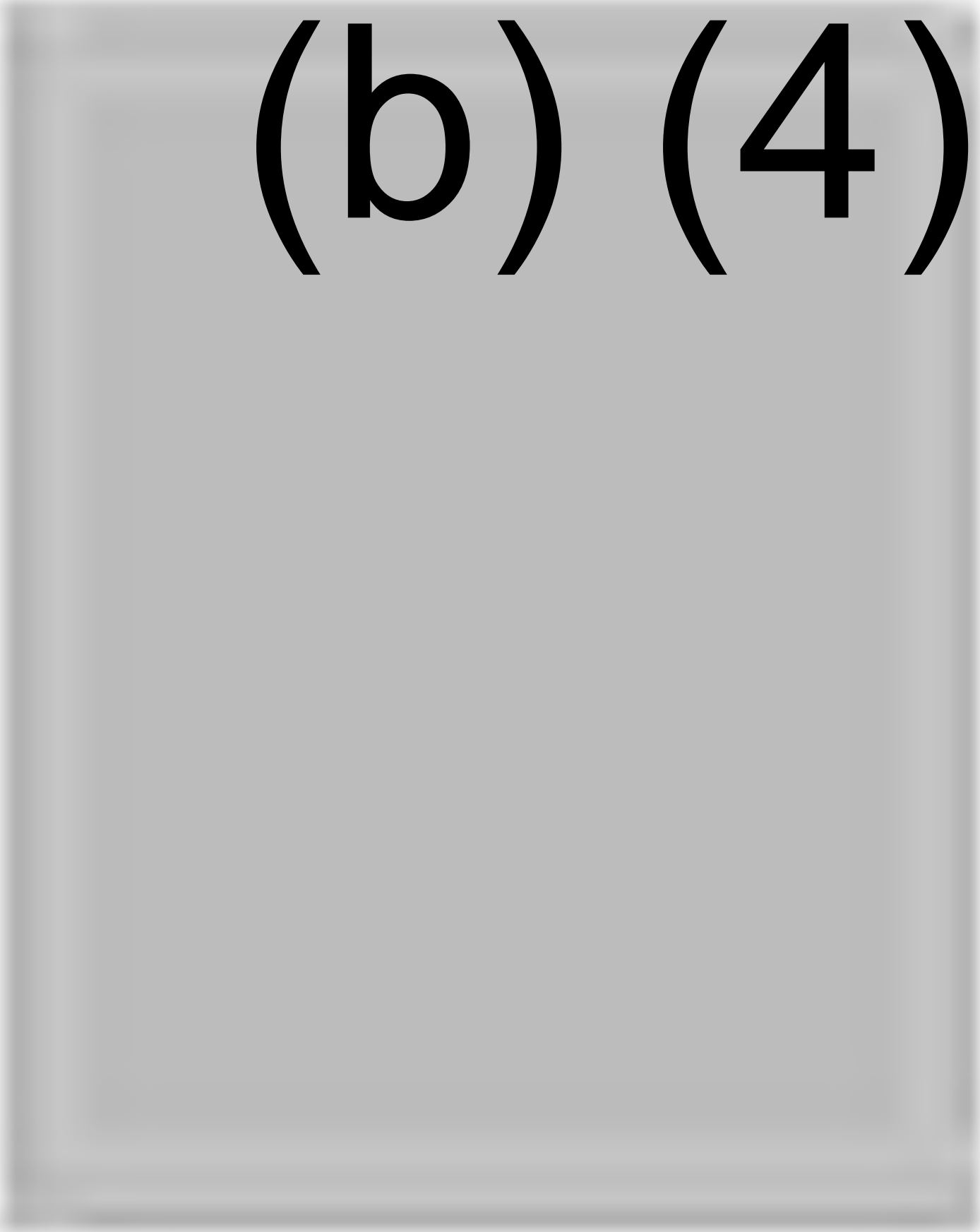
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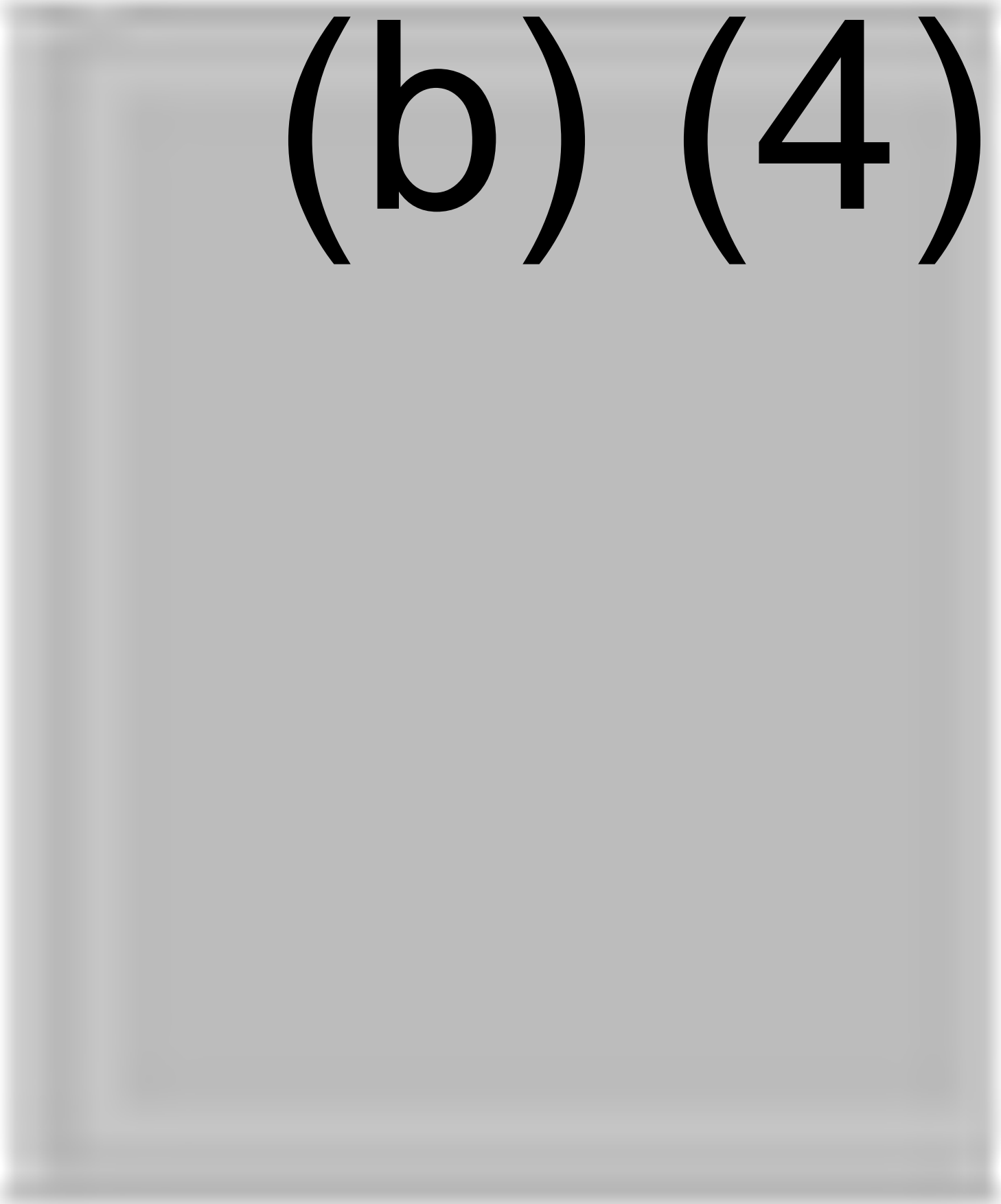
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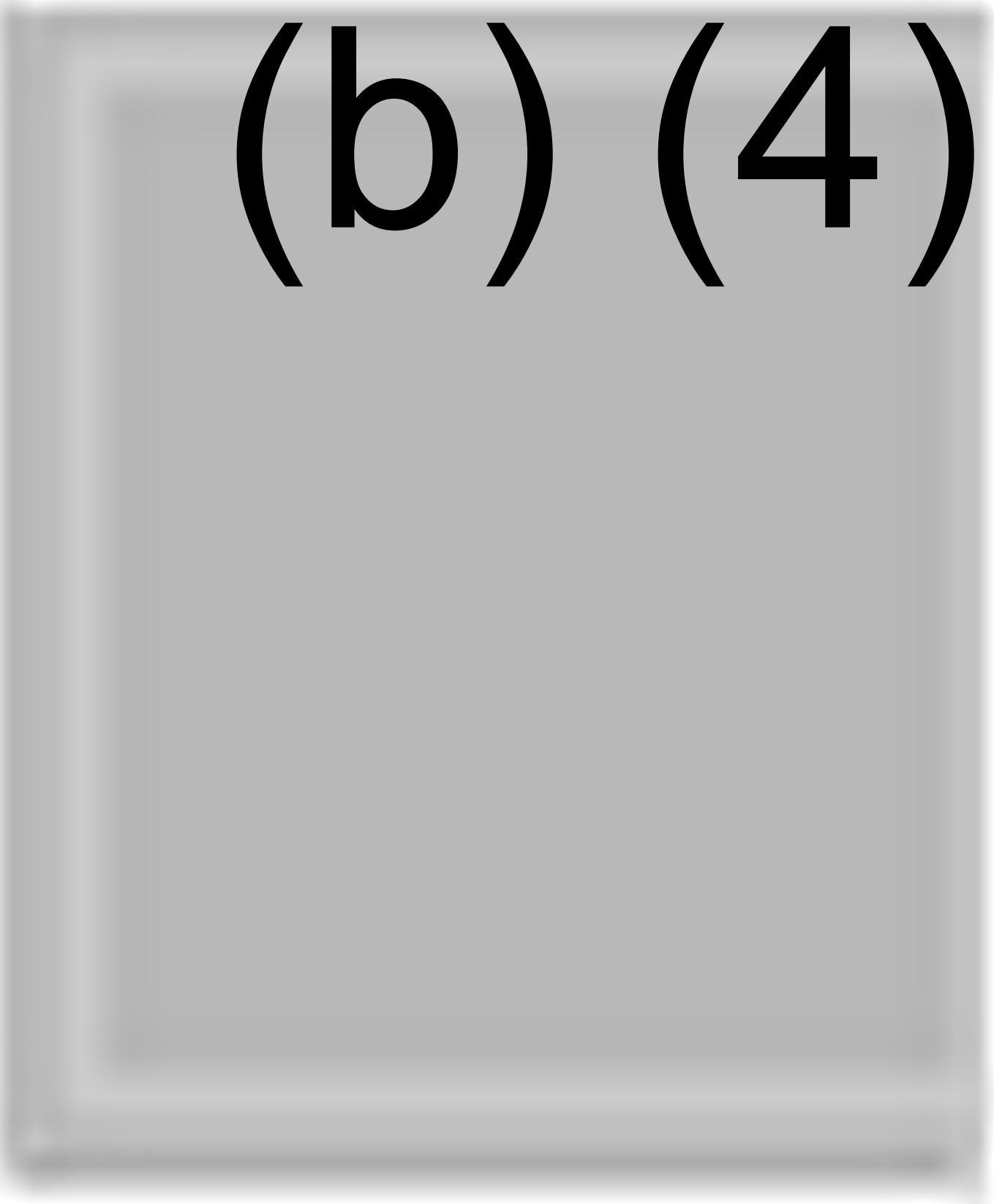
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Protocol – (b) (4)

Production Performance of Galaxis 2.0

(b) (6), (b) (4)

(b) (6)

June 15, 2021

(b) (6), (b) (4)

Date

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6/17/2021

Dr. Mallory Embree
Sponsor Representative Native Microbials

Date

1 Project Description

This research will be conducted at the (b) (6), (b) (4) . All cows will be housed in a naturally ventilated barn (see appendix A) in freestall pens with sand bedding.

2 Experimental Design

2.1 Animals and Treatments

One hundred and sixty Holstein dairy cows, between 40-160 days in milk will be allocated to a total of ten pens containing 16 cows each and pens assigned to one of two treatments (n = 5 pens/trt; Control and Galaxis 2.0). Animals should be evaluated for soundness and removed before beginning of the trial if have feet or leg issues, are a three quartered animal or had more than one case of mastitis during calving to beginning of covariate period.

2.1.1 Blocking

Assignments to pens will be stratified by days in milk. Treatments will be balanced for parity and current milk yield prior to assignment to pen.

Parity

- Primiparous [no more than 25% (n = 40; 4 per pen) of animals]
- Multiparous (2nd and greater)

Days in milk

- Animals should be within a range of 30 days in milk across pens. The closer the better

Level of milk production

- Across pens, average milk should be a tight as possible but goal should be within a range 20 lbs of milk, if possible.

Reproductive status

- (Bred, P or OP) if past 80 days in milk.

Cows will be adapted to pens for one week and then baseline data (covariate) will be collected for 2 weeks prior to start treatments for all cows. Cows will be fed their respective treatment diets for 140 days.

Treatments will consist of:

1. Control
2. Galaxis 2.0, (5 g of Galaxis 2.0/cow per d)

The treatment (5 grams per cow per day; 440 grams total per day across the 5 pens to allow for refusal) will be mixed with 4 gallons of ground corn in a 5-gallon bucket and then added on top of the dry ingredients in the mixer wagon prior to the addition of forages. Pens assigned to the control will receive a 4-gallon bucket of corn using a different bucket. Treatments will be color coded and marked on the pens. Cows will wear color-coded neck chains that will differ by pen in order to help maintain pen integrity on a daily basis.

Native Microbials will supply treatments in daily packets of approximately 400 g for (b) (4)

2.1.2 Treatments

2.1.2.1 Handling of Packets

- Treatments will be packed into a daily packet of approximately 440 g for (b) (4), with each packet containing enough product for the 5 treated pens. Packets should be stored at 4°C at (b) (4)
- Each day, a fresh packet will be opened and the contents mixed into approximately 4 gallons of ground corn in a 5 gallon bucket.
- Approximately every 50 days, one unused packet will be sent to Native Microbials for assay. Shipments should be sent on Monday or Tuesday via overnight with ice packs (Native Microbials will provide payment method).
- Ship to (b) (6) 10255 Science Center Drive, Suite C2, San Diego CA 92121-1117. Phone: (b) (6)

2.1.3 Feeding

The basal diet will be formulated to meet or exceed dairy NRC 2001 nutrient requirements and will be formulated using corn silage, alfalfa/grass haylage and concentrate mix. The control and treatment TMR will be mixed in a vertical TMR mixer and delivered to the appropriate pens in the freestall barn. Animals will be given all of their daily allotment of feed at one feeding. Animals will be fed at a reasonably consistent time each day. Individual pen feeding amounts will be adjusted daily to allow for a 5 to 10% feed refusal rate. Cow feeding areas are separated using polycarbonate barriers separating the feedbunk between each pen.

3 Observations

3.05 Dry Matter Intakes

Dry matter intakes from -21 to 140 days of treatment (daily/weekly). Diets will be offered at ad libitum intake with 5 to 10% refusals. Orts will be weighed daily by (b) (4) personnel and daily intakes calculated for the duration of the study.

3.10 Body Weight(s)

Double body weights (weights measured on two consecutive days) will be collected at beginning and end of covariate period, every 28 days and at removal from trial. Average body weight change will be calculated by 28-day periods and overall body weight change based on body weight at end of covariate

3.15 Body Condition Score (BCS)

BCS:1-5 (b) (4) scoring system: Two scorers at beginning and end of covariate, every 28 days and at removal from experiment. Average BCS will be determined and used for analysis. If BCS is ≥0.5 between scorers, then scorers will independently rescore animal.

3.20 Milk Composition

Once weekly during the trial, milk samples will be collected from each animal at each milking during a 24-hour period. Milk samples will be collected on the same day(s) of the week throughout trial. Milk samples will not be composited but will be sent to (b) (4) laboratory at (b) (4) for analysis of milk fat, protein, lactose, solids not fat, total solids, somatic cell counts, MUN and fatty acid analysis (de novo, mixed, preformed).

3.25 Milk Production

Cows are milked daily at approximately 0700, 1500, and 2300 h in a D-16 parallel milking parlor. Daily milk weights will be captured by (b) (4) software ((b) (4) software. A milking system maintenance including calibration of the meters will be performed prior to start of trial. Average daily milk and ECM yields by week and total treatment period will be calculated based on milk and milk composition data collected on the trial.

3.30 Feed Efficiency

Average daily milk and ECM by week and total treatment period will be calculated based on milk and milk composition data collected on the trial. Weekly feed efficiency will be calculated as milk/DMI and ECM/DMI.

3.35 Rumination monitoring

Daily rumination times will be measured on each cow by use of a wearable sensor ((b) (4) (b) (6)

3.40 Rumen sampling

On one day during the covariate period and again at 70 and 100 d of treatment, rumen contents will be sampled by stomach tube from 10 cows assigned to each treatment (2 cows per pen for each treatment). Samples will be processed and shipped according to protocols provided by Native Microbials.

3.45 Feed Sampling

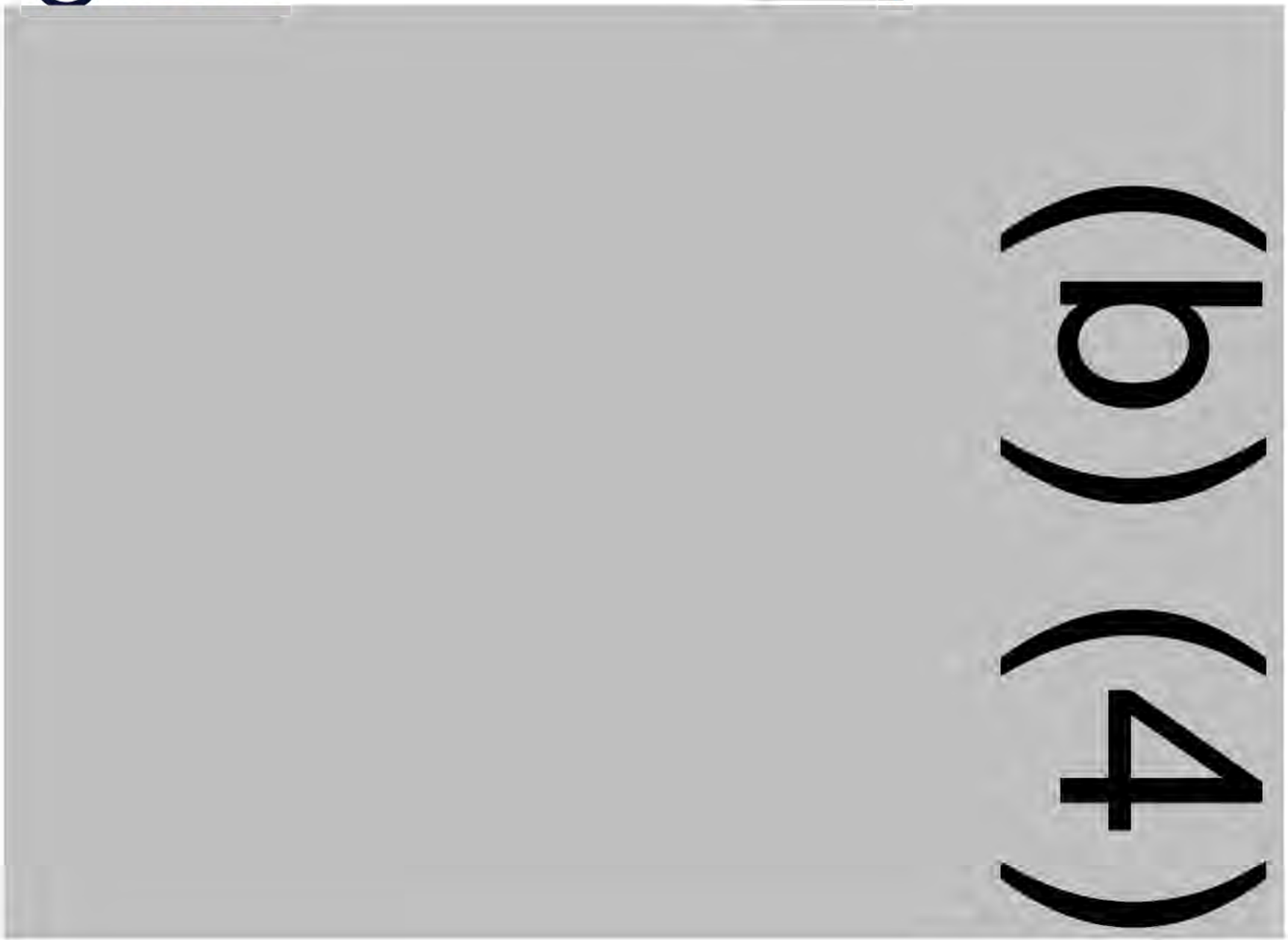
Weekly silage and TMR samples will be collected, dry matter determinations will be conducted by oven drying to constant weight, and results will be used to adjust diets for DM content of forages and for weekly calculation of DMI. Silage and TMR samples will be composited at 4-wk intervals. A sample of the basal TMR will be analyzed on a biweekly basis by NIR at (b) (4) using the (b) (4) package (b) (6)). TMR samples will be collected weekly, composited monthly and analyzed by (b) (4) ((b) (6) for the wet chemistry (b) (4) package (b) (6) Whole-trial composite samples of forages and concentrate mixtures will be analyzed for the wet chemistry (b) (4) package (b) (6).

3.50 Health and Reproduction

All health (including mastitis) and reproductive events and treatments will be captured in (b) (4) system throughout the trial and summarized by treatment. The experiment will use the approved herd health plan for the (b) (4), as well as the standard operating procedures for on-farm treatments. The dairy health program was reviewed by and approved by the (b) (4). All reproductive events will be captured in (b) (4) system.

4.0 Data/reporting

Spreadsheets will be maintained to record all data. Raw data in spreadsheet form will be provided to Native Microbials following the covariate period and at 4-week intervals throughout the study.



(b) (4)

(b) (4)

Product Comp (b) (4) (b) (4) High decrease Molasses

Product Code		(b) (4)	
Product Name		(b) (4)	(b) (4)
Formulation Name	M06091	(b) (4)	(b) (4) RUM

Product Inclusion	12.42 (lbs)

Ingredient	Amt, lbs	As Fed %
AMINO PLUS BLK	3.900	31.398
SOYBEAN HULLS	2.300	18.517
Chocolate Dairy Mix	1.284	10.340
CORN GERM MEAL	1.000	8.051
BLOOD MEAL	0.892	7.185
Sodium Sesquicarbonate	0.700	5.636
CALCIUM CARB	0.563	4.533
Whey Permeate--Tote	0.450	3.623
SALT	0.280	2.254
Fat - (b) (4)	0.250	2.013
Molasses - Blender (ML)	0.248	2.000
UREA	0.150	1.208
CALCIUM SULFATE BULK	0.118	0.952
MAG-OX 54 BULK	0.111	0.891
Smartamine M	0.060	0.483
MONO-DICAL PHOS	0.051	0.412
SELENIUM .06%	0.024	0.191
NE Dairy TM Low CU	0.017	0.133
Dairy ADE AI/MA	0.012	0.098
POT/MAG/SULFATE	0.006	0.046
(E) 90.7 RUMENSIN 90 (90.7 g/lb) (b) (4)	0.005	0.036
VIT-E 227M U/LB	0.000	0.002
Total	12.421	100.000

Nutrient	DM %	As Fed %
Forage Products, %	0.031	0.028
Fat, %	4.747	4.280
Adjusted Protein, %	32.088	28.927
Nel Dairy, mcal/cwt	76.841	69.270
NFC, %	24.972	22.512
Rumen Sol Sugar, %	12.701	11.449
Adj Tot Starch, %	4.619	4.163
Organic Acid, %		
NDF, %	19.709	17.767
Digestible NDF, %	14.419	12.998
DigNDF/NDF, ratio	0.732	0.732
uNDF 240, %	3.218	2.901
peNDF, %		
peuNDF240, %		
Calcium, %	2.608	2.351
Phosphorus, %	0.486	0.438
Sulfur, %	0.457	0.412
Magnesium, %	0.764	0.689
Potassium, %	1.283	1.157
Sodium, %	2.949	2.659
Chloride, %	1.681	1.516
DCAD, meq/100g	85.097	76.712
Copper, mg/kg	46.352	41.785
Manganese, mg/kg	228.318	205.820
Cobalt, mg/kg	5.138	4.632
Iodine, mg/kg	4.507	4.063
Zinc, mg/kg	268.354	241.912
Added Se, mg/kg	1.272	1.147
Vitamin A, kiu/lb	14.088	12.700
Vitamin D, kiu/lb	2.706	2.439
Vitamin E, IU/lb	52.405	47.241
Monensin, g/ton	73.232	66.016

(b) (6), (b) (4)

(b) (4)

Diet Summary (b) (6) (b) (4) NativeMicrob

Diet: Res. (b) (4) NatMicr

Animal					
Breed	Central US Holstein(3)	**Body Weight**	1,550	**Class**	Lactating
Number of Animals	60	**Days in Milk**	150	**Subclass**	Milking

Economics	**As Fed**		**As Fed**
Milk Price	$ 16.00	**Milk Revenue**	$ 15.20
Cost/100 lb Milk	$ 5.96	**Total Feed Cost**	$ 5.66
Purch. Cost/100 lb Milk	$ 4.88	**Purchased Cost**	$ 4.64
IOFC/100 lb Milk	$ 10.04	**IOFC / Animal**	$ 9.54

Feasible

Ingredient	**As Fed, lbs**	**DM, lbs**	**Cost $/Ton**
CS Bk2 27/33/38 04221	94.99	25.54	0.00
HCS Bk4 20/19/44 04221	35.54	7.15	0.00
Total Forage	130.53	32.69	
Corn Meal $180 92PS	11.38	9.80	180.00
Soybean Meal $465	3.81	3.35	465.00
(b) (4) (b) (4)	12.41	11.14	605.00
Total Concentrate	27.60	24.29	
Total Ration	158.12	56.98	

Nutrient	**DM Amount**	**DM Conc**	**Nutrient**	**DM Amount**	**DM Conc**
Forage Products	32.692 Lb	57.375 %	*Sulfur*	59.593 g	0.231 %
Fat	2.070 Lb	3.634 %	*Magnesium*	90.590 g	0.351 %
Adjusted Protein	9.583 Lb	16.818 %	*Potassium*	372.486 g	1.441 %
Nel Dairy	43.784 mcal	76.842 mcal/cwt	*Sodium*	153.119 g	0.592 %
NFC	25.125 Lb	44.095 %	*Chloride*	136.165 g	0.527 %
Rumen Sol Sugar	2.614 Lb	4.588 %	*DCAD*	8,643.959 meq	33.444 meq/100g
Adj Tot Starch	16.053 Lb	28.174 %	*Copper*	387.805 mg	15.005 mg/kg
Organic Acid	3.542 Lb	6.217 %	*Manganese*	1,762.739 mg	68.202 mg/kg
NDF	16.290 Lb	28.589 %	*Cobalt*	27.557 mg	1.066 mg/kg
Digestible NDF	7.731 Lb	13.568 %	*Iodine*	23.613 mg	0.914 mg/kg
DigNDF/NDF	0.475 ratio	0.475 ratio	*Zinc*	1,899.182 mg	73.482 mg/kg
uNDF 240	4.302 Lb	7.550 %	*Added Se*	6.454 mg	0.250 mg/kg
peNDF	11.901 Lb	20.886 %	*Vitamin A*	169.750 KIU	2.979 kiu/lb
peuNDF240	3.515 Lb	6.169 %	*Vitamin D*	30.267 KIU	0.531 kiu/lb
Calcium	207.271 g	0.802 %	*Vitamin E*	703.743 IU	12.351 IU/lb
Phosphorus	93.737 g	0.363 %	*Monensin*	409.549 mg	14.375 g/ton

Results

Energy Milk	93.301 lb	*Metabolizable Protein*	3,471.850 g	*MPB (Bypass)*	1,375.258 g
AA Milk	98.466 lb	*MPE (Energy)*	2,159.068 g	*Feed Efficiency (Milk/DMI)*	1.637
AA Index	109.350	*MPN (Nitrogen)*	2,096.592 g		

(b) (6), (b) (4)

evaluated, rumen fluid from dairy cows receiving NTK increased gas and VFA production from NDF degradation in vitro.

Key Words: in vitro gas, fiber

(b) (6), (b) (4)

2367W Essential oils manipulated rumen fermentation in lactating dairy cows. A. Van De Kerchhove[1], A. Delaquis[2], T. Steen[3], F. Mueller[4], and A. Park*[5], [1]*Federated Co-Op Limited, Saskatoon, SK, CA,* [2]*Sollio Agriculture, Montréal, Quebec, CA,* [3]*Tennessee Farmers Cooperative, La Vergne, TN,* [4]*Kalmbach Feeds, Inc., Upper Sandusky, OH,* [5]*Cooperative Research Farms, Richmond, VA.*

The trial objective was to determine the impact of cinnamaldehyde (CIN), carvacrol (CAR), and Oleobiotec (OLEO) on production and ruminal fermentation in lactating dairy cows. Four fistulated and 4 nonfistulated multiparous Holstein cows at 108 d in milk were utilized in a double Latin square design with 28 d periods. The cows were housed in tie-stalls and individually fed control (CON), CIN (1g/d), CAR (1g/d) and OLEO (1g/d) diets ad libitum each day. Essential oils were from PHODÉ, France. Cows were milked (2X / d) and sampled weekly for milk composition. Individual dry matter intake, milk yield, and composition were averaged by week. Cow body weight (BW) and body condition score (BCS) were evaluated twice per period. Cow ruminal samples were collected twice in each period for pH, ammonia, α amino nitrogen (AAN), peptides, volatile fatty acids (VFA), and lactate. Samples were collected during wk 4 in each period for blood urea nitrogen (BUN), β hydroxybutyrate, nonesterified fatty acid, aspartate amino transferase, and albumin) and total-tract digestibilities. Data were analyzed with PROC GLIMMIX of SAS with differences noted at $P < 0.05$ and trends at $P < 0.15$. No differences were noted for production, BW, BCS. or plasma parameters. Ruminal pH was depressed ($P < 0.005$) by all essential oils (5.87 for CON versus mean of 5.73, SEM = 0.03). Ruminal deamination appeared to be inhibited in cows fed CIN versus CON due to higher levels of peptides (0.68 *m*M, $P < 0.015$, SEM = 0.21). Feeding CIN or OLEO yielded higher propionate (3.17 and 2.09 *m*M, $P < 0.001$, SEM = 0.69, respectively) and tended to increase acetate concentrations (3.62 and 5.31 *m*M, $P < 0.07$, SEM = 1.51, respectively) compared with CON (71.63 and 23.97). In addition, CIN and OLEO had higher branched-chain (0.54 and 0.59 *m*M, $P < 0.009$, SEM = 0.11) and total VFA (7.79 and 8.13 *m*M, $P < 0.04$, SEM = 2.45) concentrations over CON. Carvacrol tended to depress total-tract acid detergent digestibility (48.19 versus 50.80%, $P < 0.03$, SEM = 0.79) compared with CON. Additional research looking into the interaction between degradable or undegradable protein and essential oils should be evaluated.

Key Words: essential oils, digestibility, fermentation

2368W Effects of exogenous amylolytic or cellulolytic enzymes inclusion on in vitro fermentation of lactating dairy cow diets in a dual-flow continuous culture system. J. R. Vinyard*[1], A. Ravelo[2,1], E. Sarmikasoglou[1], H. F. Monteiro[3,1], J. A. Arce-Cordero[1], M. L. Johnson[1], B. C. Agustinho[4,1], R. R. Lobo[1], M. G. Yungmann[1], A. H. R. Winter[1], L. M. Gilbertson[1], M. P.L Soltis[5,1], K. D. Klanderman[6], L. F. Ferraretto[7], A. P. Faciola[1], [1]*University of Florida, Gainesville, FL,* [2]*University of Minnesota, St. Paul, MN,* [3]*University of California–Davis, Davis, CA,* [4]*University of Idaho, Moscow, ID,* [5]*University of Tennessee, Knoxville, TN,* [6]*Adisseo USA Inc., Alpharetta, GA,* [7]*University of Wisconsin–Madison, Madison, WI.*

The objective of this study was to determine the effects of including different exogenous amylolytic or cellulolytic enzymes in a diet for high-producing dairy cows on in vitro ruminal fermentation. Eight dual-flow continuous culture fermenters were used in a replicated 4 × 4 Latin square. The treatments were control (CON), a xylanase and glucanase mixture (T1), an α-amylase mixture (T2), or a xylanase, glucanase, and α-amylase mixture (T3). Treatments were included at a rate of 0.008% of diet DM for T1 and T2 and 0.02% for T3 and all treatments replaced SBM compared with CON. All diets were balanced to have the same nutrient composition (30.2% NDF, 16.1% CP, and 30% starch; DM basis) and fermenters were fed 106 g/d divided into 2 feedings. At each feeding T2 was pipetted into the respective fermentern, as T1 and T3 were included in the fed diet. Experimental periods were 10 d (7 d adaptation and 3 d sample collection). Composite samples of daily effluent were collected and analyzed for VFA, NH_3-N, and lactate concentration, digestibility of DM, OM, NDF, CP, and starch, and flow and metabolism of N. Samples of ruminal content were collected from each fermenter at 0, 1, 2, 4, 6, and 8 h after feeding to determine kinetics of pH, NH_3-N, lactate, and VFA concentration over time. All data were analyzed using PROC GLIMMIX of SAS and the repeated variable of time was included for kinetics measurements. There was no effect of treatment on the mean pH, digestibility, N flow and metabolism or the concentrations of any VFA, NH_3-N, and lactate in the effluent samples, nor for pH, acetate:propionate, or the concentrations of lactate, NH_3-N, total VFA, acetate, propionate, butyrate, iso-butyrate, valerate, or cap-

(b) (4)

May 5, 2022.

SAFETY ASSESSMENT OF NATIVE MICROBIAL'S DIRECT FED MICROBIAL STRAINS

PANEL'S QUALIFICATIONS AND EXPERTISE

Bradley J. Johnson, Ph.D. Dr. Bradley J. Johnson is currently the Gordon W. Davis Regent's Chair in Meat Science and Muscle Biology and a Professor in the Davis College of Agricultural Sciences and Natural Resources' Department of Animal and Food Sciences at Texas Tech University. Johnson has been in this position since June 1, 2008. Johnson received his B. S. in Animal Sciences from South Dakota State University. He received both a M.S. and Ph.D. in Animal Sciences from the University of Minnesota. Dr. Johnson has over 30 years of research experience working in the area of growth and development and ruminant nutrition. The majority of Dr. Johnson's research over this time has involved evaluating the mechanism of action and physiology of two classes of veterinary drugs approved for meat production, steroidal implants, and β-adrenergic agonists. Many models have been used by Dr. Johnson to evaluate the mode of action of both of these veterinary drugs including cell culture, tissue explant and in vivo experiments. More recently, he has been asked to address the proposed metabolism of these compounds as it relates to potential residues in edible tissues. Dr. Johnson is currently a member of the Joint Expert Committee of Food Additives (JECFA) of the United Nation's FAO. This committee is instrumental in risk assessment of various feed additives and growth enhancing compounds used in animal production for human food consumption. Finally, Dr. Johnson has been involved in many natural feed additive research trials involving various yeast and direct-fed microbial products.

T. G. Nagaraja, BVSc, MVSc, PhD is a University Distinguished Professor and the Roy Walter Upham Endowed Professor in the Department of Diagnostic Medicine and Pathobiology in the College of Veterinary Medicine at Kansas State University, Manhattan, Kansas. His appointment carries responsibilities in research (60%), teaching (30%) and directed and non-directed services (10%). He has over 30 years of research experience in the field of Rumen Microbiology and Food Safety. His research has focused primarily on role of rumen microbes in function and dysfunction of the rumen, and on food borne pathogens, particularly Shiga toxin-producing *Escherichia coli* and *Salmonella* in cattle. His teaching responsibilities include Veterinary Bacteriology and Mycology course for the sophomore DVM students, Ruminant Digestive Physiology for the Freshman DVM students, and two courses on the rumen, Metabolism and Microbiology, for the graduate students in Ruminant Nutrition. His research has focused on the use of ionophore and other antibiotics in cattle; causes, pathogenesis, and vaccine development for liver abscesses in feedlot cattle; causes and preventions of ruminal disorders, such as acidosis and bloat; ecology of Shiga toxin-producing *Escherichia coli* and *Salmonella* in cattle; and on antimicrobial resistance and use of antimicrobial alternatives to replace antibiotics.

Jhones O. Sarturi, Ph.D. received his D.V.M. from the University for the Development of Pantanal – Brazil (UNIDERP), a M.S. degree in Agronomy from the University of Sao Paulo Brazil (USP/ESALQ), a Ph.D. in Animal Science from the University of Nebraska Lincoln (UNL) and worked as a Post-Doctoral Research Associate at Texas A&M AgriLife Research, Amarillo - Texas. Currently, a tenured faculty (Associate Professor) at Texas Tech University, Department of Animal and Food Sciences (Davis College of Agricultural Sciences and Natural

Resources), with a research/teaching/service appointment. Research focus on beef cattle nutrition and ruminal metabolism, which involves the development of strategies to improve, evaluate, and better utilize byproducts, forages, and grains in ruminant diets. Dr. Sarturi's research approach involves the manipulation of nutrients/molecules at pre and/or post animal consumption. Research endeavors had involved the development, assessment, and application of live microorganisms to ruminant diets. His additional responsibilities involve, but are not limited to: a) manager for the Ruminant Nutrition Laboratory (campus) and the Ruminant Nutrition Center (cattle metabolism area); b) primary representative for the Department of Animal and Food Sciences at the Institutional Animal Care and Use Committee (IACUC); and teaching c) undergraduate (Feeds and Feeding; Stocker Cattle & Feedlot Management) and graduate-level courses (Research Methods in Ruminant Nutrition; Minerals and Vitamins in Animal Nutrition; Advanced Feedlot Management; and Nutrition Seminar). For additional qualifications and/or contact, please use the link as follows: https://www.depts.ttu.edu/afs/people/faculty_sarturi.php

SAFETY ASSESSMENT

The panel, convened to conduct a risk assessment, consistent with the requirements as provided by the Office of Texas State Chemist Memoranda 5-21, for the safety of two direct fed microbial dairy products intended for marketing in Texas, reviewed the documents provided by Native Microbials, San Diego, CA. on four microbial strains (3 bacteria and 1 yeast) for use as direct-fed microbials for cattle. The four microbial strains are: *Butyrivibrio fibrisolvens*, strain ASCUSDY19, *Clostridium beijerinckii*, strain ASCUSDY20, *Ruminococcus bovis*, strain ASCUSDY10, and *Pichia kudriavzevii*, strain ASCUSDY21. The four microbial strains are present in two commercial products, Galaxis® and Galaxis Frontier®, to be marketed by the company. The Galaxis® contains *P. kudriavzevii* and *C. beijerinckii*, while the Galaxis Frontier® is composed of all four microbial strains. Two of the three bacterial strains and the yeast strain are commercially presented under triacylglycerol encapsulation, and the third bacterial strain (*C. beijerinckii*) is presented as unencapsulated spores and are intended to be included in dairy cow diets to provide a supplemental source of viable microbes. The four microbial strains are expected to contribute to the digestion of fiber- and starch-based diets to produce volatile fatty acids, which will be utilized as source of energy by cattle.

Our assessment of the safety of the four microbial strains for animals and humans are based on the following criteria:

1. **The four strains belong to species that are members of the normal microbial community in the rumen of dairy cattle**

   All four strains were isolated from ruminal contents of healthy Holstein cows in mid-lactation. The four strains have been unequivocally identified taxonomically at the genus and species level based on phenotypic and genotypic characteristics. The phenotypic characteristics included colony and microscopic morphology, the substrates that serve as energy source for the growth of the organisms, and the fermentation products produced. The substrates tested included a variety of sugars that are expected to be present in ruminant diets. The genotypic characterization included amplification and sequence analysis of the 16S rRNA gene and whole genome sequencing, which provides a comprehensive genetic blueprint of the microbial strains. Of the four strains, *R. bovis*, strain ASCUSDY10, is a novel strain which has not been reported in the literature. In fact, the strain was first isolated, characterized, named, and published by the researchers in Native Microbials (Gaffney et al.,

2021. International Journal of Systematic and Evolutionary Microbiology, 71(8): 004924; https://dx.doi.org/10.1099%2Fijsem.0.004924). The IJSEM is the official journal for publication of novel microbial species. Although *R. bovis* is a novel species, the genus *Ruminococcus* is a common genus and is prevalent in all cattle. The genus has two common species, *albus* and *flavefaciens*, which are dominant species in the rumen of cattle offered diets containing roughages and grains, such as in typical dairy cow operations in US.

2. **The four strains of microbial strains are closely related to the other strains of the species prevalent in the rumen of cattle**

The relatedness of the four strains were compared at the whole genome level with a number of strains of the same species. The genomic sequences were retrieved from GenBank[90]. The GenBank is the National Institute of Health genetic sequence database, an annotated collection of all publicly available DNA sequences and is part of the International Nucleotide Sequence Database Collaboration, which comprises databases from Japan, Europe and the US (National Center for Biotechnology Information; NCBI). The relatedness was determined by comparing average nucleotide identity (ANI), which is a measure of nucleotide-level genomic similarity between the two organisms. The ANI determines if two genomes belong to the same species and how closely the strains are related to each other within the species. A cutoff score of >95% indicates that the two genomes belong to the same species. The ANI values of >95% not only confirms the species, but also indicates the closeness of the strains that have been isolated, sequenced and publicly deposited by other scientists. The ANI values reported were 95% for *B. fibrisolvens*, strain ASCUSDY19, 98% for *C. beijerinckii*, strain ASCUSDY20, and 99% for *P. kudriavzevii*, strain ASCUSDY21 with the strains of the same species isolated from cattle, whole genome sequenced and deposited in the GenBank. The ANI values for *R. bovis*, strain ASCUSDY10 were not close to any of the species of the genus *Ruminococcus*, instead, the best match was an unnamed and uncultured organism in the genus *Eubacterium*, which further confirms the novelty of the organism.

3. **The four microbial strains do not contain any virulence genes that code for toxins or other independent virulence factors that may contribute to pathogenicity**

A major safety consideration of probiotic bacterial and fungal species is an assessment that they are harmless and do not have the potential to cause infection in target animals or humans handling or exposed to the products. The whole genome sequences available allows assessment of the pathogenic potential for the four microbial strains. All publicly available pathogen and virulence-based databases (PATRIC database, virulence factors database [VFDB] and the PATRIC_VF database) reported were queried to determine pathogenic potential of the four microbial strains. In total, these databases encompass 138,461 known pathogen-related genes and represent 331,756 bacterial genomes. The alignment process compared all identified genes in the four microbial strains against all known pathogen-related genes that have been identified across the bacterial and fungal kingdoms. In addition, PathogenFinder and IslandViewer web servers and BLASTp alignment to the Pathogen-Host Interaction Database (Phi-Base) were searched to assess the pathogenicity and virulence. The Pathogenfinder model predicts pathogenicity based on matches to proteins found differently in pathogenic and nonpathogenic bacteria. The search for virulence and pathogenic genes in *P. kudriavzevii*, strain ASCUSDY21 involved all potential nomenclature due to previous classification of the genus *Pichia*, which used to be the largest yeast genus.
The search for virulence and pathogenic genes yielded the following information:

a. A single hit for a recombinase protein, which is ubiquitous and not related to pathogenicity or virulence in *B. fibrisolvens*, strain ASCUSDY19.
b. A gene that codes for a transport protein (TrSE) in the genome of *R. bovis*, ASCUSDY10. Such a membrane protein is also present in pathogenic as well as non-pathogenic species of other ruminococci.
c. The genome of *C. beijerinckii*, strain ASCUSDY2 do not contain any genes that encode for toxins commonly associated with *Clostridium* species.
d. Comprehensive alignment of *P. kudriavzevii*, strain ASCUSDY21 genome to the databases yielded twenty-three hits at 80% identity. Further investigation of the alignments revealed no genes directly involved in pathogenesis or toxin production. Genes that aligned in the databases were either structural or related to general cell function.

Pathogenicity islands, which are a cassette of genes that encode for virulence factors typically associated with pathogens, were not present in any of the four microbial strains Plasmids, which are extrachromosomal DNA in microorganisms and often carry genes that encode for virulence factors or antimicrobial resistance, were not detected in *C. beijerinckii*, strain ASCUSDY20, *R. bovis*, strain ASCUSDY10, and *P. kudriavzevii*, strain ASCUSDY21. *Butyrivibrio fibrisolvens*, strain ASCUSDY19 contained a chromid (336, 856 bp), which is neither a chromosome nor a plasmid. The presence of chromid is consistent with the reports of its presence in other strains of *B. fibrisolvens*. The annotated features on the chromid were associated with general housekeeping and metabolic functions. No genes encoding for toxins, other virulence factors or antimicrobial resistance were detected on the chromid.

4. **The four microbial strains are not likely to contribute to the antimicrobial resistance (AMR) of bacteria or fungi in the gastrointestinal tract of cattle or in the environment**

It is important that direct fed microbial products containing viable microbes do not contribute to the pool of antimicrobial resistance (AMR) genes, particularly to medically important antimicrobials, already present in the gut microbial population. In bacterial and yeast species, resistance to certain antimicrobials is inherent and is typical of all the strains of that species. Inherent resistance is not considered a safety concern. In contrast, when a strain that is typically susceptible becomes resistant to an antibiotic, it is because of acquired resistance. The susceptibility and resistance of the four microbial species were determined *in vitro* (phenotypic testing) and *in silico* (genotypic testing) by interrogating the whole genomes for the presence of AMR genes.

For the three bacterial strains, phenotypic testing was conducted to determine the minimum inhibitory concentrations (MIC) against a selected antimicrobials relevant to human and veterinary medicine. The results were evaluated against the resistant breakpoints set by the European Food Safety Authority (EFSA) for "other Gram-positive bacteria" and fungi, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "Gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" and fungi. The MICs obtained for *P. kudriavzevii* ASCUSDY21 were compared with available epidemiological cut-off values (ECOFF) and breakpoints. The CLSI breakpoints for *Candida* species were used. The genotypic analysis for AMR was based on the analyses of the whole genome sequences for the presence of AMR genes. The amino acid sequences from coding regions were aligned to the PATRIC database, which includes the Comprehensive Antibiotics Resistance Database CARD) and NCBI's National Database of Antibiotic Resistant Organisms (NDARO). In addition, AMR was further explored using the

ResFinder web server and BLASTp alignment to the NCBI AMR database as used by AMRFinder. These databases included a total of 30,748 protein sequences.

All three bacterial strains were susceptible to the group of antibiotics assessed, and the yeast, *P. kudriavzevii,* was susceptible to antifungal antibiotics based on the MIC values, with a few exceptions. The MIC values for *B. fibrisolvens* ASCUSDY19 for tetracycline, gentamycin, kanamycin, streptomycin, and erythromycin were higher than breakpoints established by EFSA. Resistance to gentamycin, kanamycin, and streptomycin is expected (intrinsic resistance) because they belong to the class aminoglycosides, whose spectrum of activity is restricted for aerobic Gram-negative bacteria (*B. fibrisolvens* is an anaerobic Gram-positive bacteria). The WGS analysis indicated the presence of *tetW* gene, which confers resistance though ribosomal protection. The MIC values for *C. beijerinckii,* ASCUSDY20 to all antibiotics assessed were lower than the CLSI breakpoints, which means they are susceptible. The antibiotic susceptibility and resistance profiles of *R. albus,* ASCUSDY10 were similar to *B. fibrisolvens* ASCUSDY19. Intrinsic resistance was detected for aminoglycosides and resistance to tetracycline was because of the presence of *tetW* gene in the genome. The MIC testing of *P. kudriavzevii,* ASCUSDY21 indicated the organism was susceptible to the antifungal antibiotics.

Overall, the antimicrobial susceptibility data provided suggest that it is unlikely that the four microbial strains will contribute to the pool of antibiotic resistant organisms in the gut of dairy cattle or in the environment. The only exception was resistance of *B. fibrisolvens* ASCUSDY19 and *C. beijerinckii* ACUSDY20 to tetracycline, a medically important antibiotic. The gene responsible for the resistance was on the chromosome, therefore not likely to be transferred horizontally to other bacteria. Tetracycline resistance is widespread among many ruminal bacteria because of the use of tetracyclines in the cattle production systems for more than 70 years. Also, none of the four microbial strains contained plasmid, which suggest that it is unlikely the four microbial strains will be involved horizontal gene transfer of AMR genes.

5. **Safety of the four microbial strains is supported by studies conducted at universities that have evaluated their impact on milk production in dairy cows**

Two independent studies conducted by university researchers, one at (b) (6) (b) (6) which investigated the effects of feeding the two commercial products, Galaxis® and Galaxis Frontier®, on feed intake and milk production provide evidence for the safety of the four microbial strains. Both studies have been peer-reviewed and accepted for publications in the Journal of Dairy Science Communications, an official journal of the American Dairy Science Association. The study conducted at (b) (4) was on Galaxis® and the study at the (b) (4) (b) (4) included both products. Each study had a control group that did not receive any microbial products. Both studies indicate that feeding of the two products had no adverse effects on feed intake, milk production, and had shown any negative health outcome.

6. **Safety of the four microbial strains is supported by assessments reported by the company that indicated no negative influence on the ruminal microbiome**

Native Microbials have conducted studies in dairy cows to determine if daily administration of *C. beijerinckii,* ACUSDY 20 or *P. kudriavzevii,* ASCUSDY21 altered the rumen microbial community composition. The study was conducted on twenty-four animals: one group of eight cows received *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 (Microbes 1), a second group of eight cows received *C. beijerinckii*

ASCUSDY20, *P. kudriavzevii* ASCUSDY21, and a third native rumen bacterium (Microbes 2), and a third group of eight cows served as the control group (No microbes). In this study, the administration of *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 to dairy cows did not significantly alter the rumen fungal or bacterial composition when compared to the control group. The average relative abundance of each phylum tended to be similar across experimental groups. Relative abundances of all fungal and bacterial phyla were within the standard ranges observed in cows not fed native rumen microbes. Therefore, directly feeding *C. beijerinckii* ASCUSDY20 and *P. kudriavzevii* ASCUSDY21 did not dramatically alter rumen microbiome, which provides additional evidence no adverse effects with the feeding of the two microbial strains.

7. **The carriers and excipients of the commercial products are authorized feed ingredients and do not raise safety issues**

   Batch tests of commercial products containing the microorganisms were safe and free from toxins and other potential pathogens. Calculation of other nutrient quantities provided by the commercial product are nutritionally irrelevant when compared to a healthy dairy cow overall diet daily consumption.

DETAILED ASSESSMENT SEPARATED BY MICROORGANISM

Keys points for the assessment involved the characterization and the genomic classification of the aforementioned **commensal ruminal microorganisms**, isolation and identification have been performed from the **rumen of healthy, mid-lactation dairy cows** via ruminal cannula. Microorganism's strains identification was supported by 16S rRNA and whole **genome analysis**, which was also used for identification of genes and proteins related to **pathogenicity and virulence**, also used in combination with an extensive literature review. The potential **antimicrobial susceptibility**, **resistance**, and potential for **production** of medically important antimicrobials were also evaluated. Published, submitted, or completed dairy studies included in the packet were assessed with focus on potential **negative health outcomes** or potential negative **disturbance on ruminal microbiome** relative abundances. **Commercial product carrier** quality, safety, and quantity of nutrients were evaluated for any potential **relevance for nutrient tolerance** for dairy cows. It was also considered the **potential effect on health outcome** that could be induced by **failure** of the ruminal microbial activity of added microorganisms. Finally, the literature review was also considered for any **potential risk for target animal health, human, and food safety** under the intended conditions of use as a direct fed microbial.

1st Individual Generally Recognized as Safe conclusion: "... ***Butyrivibrio fibrisolvens (ASCUSDY19)*** *should not be associated with any safety concerns for dairy cattle under the intended conditions of use as direct fed microbial...*" **and** "... ***Butyrivibrio fibrisolvens (ASCUSDY19)*** *should not be associated with any human food safety concerns under the intended conditions of use as direct fed microbial in the feed of dairy cattle...*". Content disclosed on PDF page number 54 of the dossier.

Connected to such microbial control susceptibility, it is critical that microorganism in question is **responsive to antimicrobial strategies.** Current GRAS dossier shows microbial susceptibility to chloramphenicol, vancomycin, and ampicillin. Although chloramphenicol is not allowed to be used in food producing animals (FDA, green book), vancomycin (usually not deemed recommended to gram–negative bacteria or approved to be used in food producing

animals in US), but the ampicillin on the other hand, is readily available in the US market. Current report shows that *Butyrivibrio fibrisolvens* (ASCUSDY19) is not susceptible to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin, although such fact is likely due to current microorganism being anaerobic rather than a resistance induced by genotype [except by tetracycline, in which a resistant gene (tetW) was identified]. The *Butyrivibrio fibrisolvens* (ASCUSDY19) was deemed as **not pursuing any microbial inhibitory activity** of medical interest, once such strain was submitted to an assessment involving multiple reference strains known to be susceptible to a range of antibiotics, and no zones of inhibition were observed.

The proposed daily consumption (5 g, as-is basis) of the **commercial product** presentation contains 30% sodium sulfate, 50% hydrogenated glycerides, and 20% freeze-dried bacterial powder [containing the *Butyrivibrio fibrisolvens* (ASCUSDY19)]. Such combination will provide daily amounts of **additional nutrients, such as sodium, sulfur, fatty acids, and glycerol**. However, given the expected daily intake of mature dairy cows (20 to 30 kg/day, DM basis), the contribution of such **additional nutrients can be considered as nutritionally irrelevant**. Current dossier also provides evidence of final product quality control, in which toxins such as botulinum were tested and deemed negative. Additional quality control within the final product batches involving the detection of pathogenic microorganisms such as ***Coliforms***, ***E.coli***, ***Salmonella***, **and** ***Listeria*** were also assessed and deemed as **negative or negligible** (safe) levels.

The dose of 1×10^8 CFU of *Butyrivibrio fibrisolvens* (ASCUSDY19)/cow-daily is consistent to other direct fed microbials currently available to the cattle industry and published in the literature. The **microbial activity failure** by the *Butyrivibrio fibrisolvens* (ASCUSDY19) in performing expected improvement in nutrient (carbohydrate) ruminal degradation and generation of metabolites (acetate, butyrate, and lactate) **should not impair the host ability to meet nutrient and energy requirements**. The dietary nutrient requirements for the expected level of production will be formulated independently from the presence of *Butyrivibrio fibrisolvens* (ASCUSDY19) or its activity. For instance, in case of complete failure in such additional microbial activity, the "inactive" microbes will simply be part of the pool of metabolizable protein delivered to the small intestine of the host. Literature review also made inferences to the additional ruminal biohydrogenation activity performed by *Butyrivibrio fibrisolvens*, which induces the synthesis of conjugated linoleic acids (*cis*-9 *trans*-11), also known as CLA's. Some isomers of CLA, such as the *trans*-10 *cis*-12, have been connected to milk fat depression, although that is not the case of the CLA originated from *Butyrivibrio fibrisolvens* ruminal biohydrogenation. More specifically, the *Butyrivibrio fibrisolvens* (ASCUSDY19) has been **offered to dairy** cows at recommended dose in current dossier (noted by at least three **publications** included in the packet), in which animals **did not show any adverse effect or signs of pathogenicity** induced by the additional live microorganism included in the diet, other than positive effects on dairy cow's productivity.

No related cases of infection or adverse effects when supplemented to cattle or human were noted in the broad literature review performed in the dossier, other than likely unrelated sporadic cases of physical injury added to a secondary microbial contamination. **Cases of gastrointestinal and hepatic infections** involving other microorganisms in which *Butyrivibrio fibrisolvens* were within reports where solely based on morphology, metabolism, and susceptibility profiles, **while causation (microbial identification) were not present**.

2nd Individual Generally Recognized as Safe conclusion: "... ***Ruminococcus bovis (ASCUSDY10)*** *should not be associated with any safety concerns for dairy cattle under the*

intended conditions of use as direct fed microbial..." **and** "... ***Ruminococcus bovis (ASCUSDY10)*** *should not be associated with any human food safety concerns under the intended conditions of use as direct fed microbial in the feed of dairy cattle...*". Content disclosed on PDF page number 133 of the dossier.

Connected to *Ruminococcus bovis* (ASCUSDY10) **microbial control susceptibility**, it is critical that the microorganism in question is responsive to antimicrobial strategies. Current GRAS dossier shows microbial susceptibility to clindamycin, chloramphenicol, vancomycin, and ampicillin. Clindamycin has been cleared by the FDA to be used in cats and dogs (FDA, green book), chloramphenicol is not allowed to be used in food producing animals (FDA, green book), vancomycin (usually not approved to be used in food producing animals in US), but the ampicillin on the other hand, is readily available in the US market. Current report shows that *Ruminococcus bovis* (ASCUSDY10) **is not susceptible to** tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin, although such fact is likely due to current microorganism being anaerobic rather than a resistance induced by genotype [except by tetracycline, in which a resistant gene (tetW) was identified]. The *Ruminococcus bovis* (ASCUSDY10) was deemed as **not pursuing any microbial inhibitory activity** of medical interest, once such strain was submitted to an assessment involving multiple reference strains known to be susceptible to a range of antibiotics, and no zones of inhibition were observed.

The proposed daily consumption (5 g, as-is basis) of the **commercial product** presentation contains 30% sodium sulfate, 50% hydrogenated glycerides, and 20% freeze-dried bacterial powder [containing the *Ruminococcus bovis* (ASCUSDY10)]. Such combination will provide daily amounts of **additional nutrients, such as sodium, sulfur, fatty acids, and glycerol**. However, given the expected daily intake of mature dairy cows (20 to 30 kg/day, DM basis), the contribution of such **additional nutrients can be considered as nutritionally irrelevant**. Current dossier also provides evidence of final product quality control, in which toxins such as botulinum were tested and deemed negative. Additional quality control within the final product batches involving the detection of pathogenic microorganisms such as ***Coliforms***, ***E.coli***, ***Salmonella***, **and** ***Listeria*** were also assessed and deemed as **negative or negligible** (safe) levels.

The dose of 1×10^8 CFU of *Ruminococcus bovis* (ASCUSDY10)/cow-daily is consistent other direct fed microbials currently available to the cattle industry and published in the literature. The **microbial activity failure** by the *Ruminococcus bovis* (ASCUSDY10) in performing expected improvement in nutrient (carbohydrate) ruminal degradation and generation of metabolites (acetate and ethanol) **should not impair the host ability to meet nutrients and energy requirements**. The dietary nutrient requirements for the expected level of production will be formulated independently from the presence of *Ruminococcus bovis* (ASCUSDY10) or its activity. For instance, in case of complete failure in such additional microbial activity, the "inactive" microbes will simply be part of the pool of metabolizable protein delivered to the small intestine of the host. The *Ruminococcus bovis* (ASCUSDY10) has been offered to **dairy cows at recommended dose** in current dossier (noted by at least three **publications** included in the packet), in which animals **did not show any adverse effect or signs of pathogenicity** induced by the additional live microorganism included in the diet, other than positive effects on dairy cow's productivity.

No related cases of infection or adverse effects when supplemented to cattle or human were noted in the broad literature review performed in the dossier. The search for literature involving the mechanisms of bacterial translocation from the digestive tract into extra intestinal sites in the body using the generic term *Ruminococcus* yielded non-relevant information.

3rd Individual Generally Recognized as Safe conclusion: "... ***Clostridium beijerinckii (ASCUSDY20)*** *spray dried powder should not be associated with any safety concerns for dairy cattle under the intended conditions of use as direct fed microbial...*" **and** "... ***Clostridium beijerinckii (ASCUSDY20)*** *spray dried powder should not be associated with any human food safety concerns under the intended conditions of use as direct fed microbial in the feed of dairy cattle...*". Content disclosed on PDF page number 194 of the dossier.

A comprehensive genomic assessment has been performed and indicates the absence of direct inference connecting *Clostridium beijerinckii* (ASCUSDY20) with pathogenic elements for the intended animal feeding purpose, for the specified dose (1×10^7 CFU/cow-daily), neither offers a human food safety concern. The search for **virulent and pathogenic genes** yielded no observations, in which **none of the predicted proteins** in the *Clostridium beijerinckii* (ASCUSDY20) genome **had any close match with homologous associated with pathogenicity, or any genes involved in toxin synthesis** (example: BoNT, botulinum neurotoxin). The toxin BoNT is popularly known as the cause for Botulism. Such toxin is closely related to *Clostridium botulinum* and in some cases related to *Clostridium butyricum*, although, no literature evidence reported in current dossier makes a correction with *Clostridium beijerinckii*. Comprehensive literature review provided support that *Clostridium beijerinckii* refers to a gram -positive, catalase and oxidase negative bacterium that readily sporulates which is **part of the natural relative abundance ruminal microbiota of the intended animals (dairy cows)**.

Connected to *Clostridium beijerinckii* (ASCUSDY20) **microbial control susceptibility**, it is critical that the microorganism in question is responsive to antimicrobial strategies. Current GRAS dossier shows microbial susceptibility to all anti-microbials, **except by** gentamycin, chloramphenicol, and tetracycline. The gentamycin resistance is not of importance, because its uptake by microorganisms involves respiration, and given that *Clostridium beijerinckii* (ASCUSDY20) is anaerobe such resistance would be expected. The resistance to chloramphenicol would be of less importance because this drug is not allowed to be used in food producing animals (FDA, green book). Its resistance is likely to be a result of a chromosomally located chloramphenicol acetyltransferase gene identified in the genetic analysis, and the absence of plasmid in *Clostridium beijerinckii* (ASCUSDY20), would make horizontal transfer very unlikely. The two tetracycline resistance genes (tetA and tetB) were identified are chromosomally located, which is indicative of natural resistance.

The *Clostridium beijerinckii* (ASCUSDY20) was deemed as **not pursuing any microbial inhibitory activity** of medical interest, once such strain was submitted to an assessment involving multiple reference strains known to be susceptible to a range of antibiotics, and no zones of inhibition were observed.

The proposed daily consumption (2.5 g, as-is basis) of the **commercial product** presentation contains 70% starch and 30% freeze-dried bacterial powder [containing the *Clostridium beijerinckii* (ASCUSDY20)]. Such combination will provide additional **1.75 g of starch daily per cow**. Given the expected daily intake of mature dairy cows (20 to 30 kg/day, DM basis), the contribution of the additional starch content can be **considered as nutritionally irrelevant**. Current dossier also provides evidence of final product quality control, in which toxins such as **botulinum** were tested and deemed **negative**. Additional quality control within the final product batches involving the detection of pathogenic microorganisms such as ***Coliforms***, ***E.coli***, ***Salmonella***, **and** ***Listeria*** were also assessed and deemed as **negative or negligible** (safe) levels.

The dose of 1×10^7 CFU of *Clostridium beijerinckii* (ASCUSDY20)/cow-daily is consistent other direct fed microbials currently available to the cattle industry and published in

the literature. The microbial **activity failure** by the *Clostridium beijerinckii* (ASCUSDY20) in performing expected improvement in nutrient (carbohydrate) ruminal degradation and generation of metabolites (acetate, butyrate, ethanol, and 1-butanol) **should not impair the host ability to meet nutrients and energy requirements**. The dietary nutrient requirements for the expected level of production will be formulated independently from the presence of *Clostridium beijerinckii* (ASCUSDY20) or its activity. For instance, in case of complete failure in such additional microbial activity, the "inactive" microbes will simply be part of the pool of metabolizable protein delivered to the small intestine of the host. The *Clostridium beijerinckii* (ASCUSDY20) has been offered to dairy cows at recommended dose in current dossier (noted by at least three **publications** included in the packet), in which animals did **not show any adverse effect or signs of pathogenicity** induced by the additional live microorganism included in the diet, other than positive effects on dairy cow's productivity. In addition, **no related cases of infection or adverse effects** of *Clostridium beijerinckii* **animals or human** were noted in the broad literature review performed in the dossier.

4th Individual Generally Recognized as Safe conclusion: "... ***Pichia kudriavzevii (ASCUSDY21)*** *should not be associated with any safety concerns for dairy cattle under the intended conditions of use as direct fed microbial.* **"** and "... ***Pichia kudriavzevii (ASCUSDY21)*** *should not be associated with any human food safety concerns under the intended conditions of use as direct fed microbial in the feed of dairy cattle...*". Content disclosed on PDF page numbers 256 and 257 of the dossier.

A comprehensive genomic assessment has been performed and indicates the absence of direct inference connecting *Pichia kudriavzevii* (ASCUSDY21) with pathogenic elements for the intended animal feeding purpose, for the specified dose (1×10^8 CFU/cow-daily), neither offers a human food safety concern. **The search for virulent and pathogenic genes** involved all potential nomenclature due to previous classification of the genus Pichia, which used to be the largest yeast genera. More recent developments in gene sequencing resulted in a more refined classification, although conservatively, all potential nomenclature were included in the search. No genes directly involved on pathogenesis or toxin production were identified. As expected, due to redundancy of entries on databases and the ubiquitous nature of the microorganism in question, twenty-three related genes yielded a match, although none of those genes are considered causative of pathogenesis. With no exception, genes were related to purine synthesis (also observed in *Vibrio Cholera*); protein kinase and peroxin-1 eommon in organelles (*Cryptococcus neoformans*); FSK1, which is a component in glucan synthase involved in cell wall synthesis (*Candida krusei*); HSP90 responsible for protein stabilization in pathogenic *Candida*, although also found in humans and other eukaryotes; actin and tubulin (cytoskeleton components)and HOG1 (kinase) which can be found in pathogenic and non-pathogenic yeasts, such as *Saccharomyces cerevisiae*; non-specific kinases (observed in pathogenic *Candida*), although not directly causative of pathogenicity or virulence; two non-specific phosphatases and one metallophosphatase, which are used for phosphate acquisition for all microorganisms; Phosphokinase, which is a protein found in pathogenic and non-pathogenic fungi and not known to cause pathogenicity or virulence; signaling molecule 14-3-3 family, which are proteins highly conserved in yeasts as well as other eukaryotes; and two histones, which are ubiquitous DNA packaging proteins.

Comprehensive literature review supports *Pichia kudriavzevii* refers to a facultative and catalase positive yeast, which is part of the natural relative abundance ruminal microbiota of the intended animals (dairy cows). Although not a limiting factor for current DFM, in case of any potential opportunistic secondary infection by *Pichia kudriavzevii* (ASCUSDY21), the

microorganism can be controlled due to its **antimicrobial susceptibility**. Current GRAS dossier shows microbial susceptibility to anidulafungin (approved for humans), amphotericin (approved for humans), micafungin (approved for humans), caspofungin (approved for humans), 5-flucytosine (approved for humans), posaconazole (approved for dogs), voriconazole (approved for humans), itraconazole (approved for cats), and fluconazole (approved for humans). Current report shows that *Pichia kudriavzevii* (ASCUSDY21) contains a gene homologue to FSK1, which is known to cause **resistance to antifungal drugs** in the group of echinocandins, however, it also lacks the required mutations necessary to induce such resistance. The *Pichia kudriavzevii* (ASCUSDY21) was deemed as **not pursuing any microbial inhibitory activity** of medical interest, once such strain was submitted to an assessment involving multiple reference strains known to be susceptible to a range of antibiotics, and no zones of inhibition were observed.

The proposed daily consumption (2.5 g, as-is basis) of the **commercial product** presentation contains 30% sodium sulfate, 50% hydrogenated glycerides, and 20% freeze-dried bacterial powder [containing the *Pichia kudriavzevii* (ASCUSDY21)]. Such combination will provide daily amounts of additional nutrients, such as **sodium, sulfur, fatty acids, and glycerol**. However, given the expected daily intake of mature dairy cows (20 to 30 kg/day, DM basis), **the contribution of such additional nutrients can be considered as nutritionally irrelevant**. Current dossier also provides evidence of final product quality control, in which toxins such as botulinum were tested and deemed negative. Additional quality control within the final product batches involving the detection of pathogenic microorganisms such as ***Coliforms, E.coli, Salmonella,* and *Listeria*** were also tested and deemed as **negative or negligible** (safe) levels.

The dose of 1×10^8 CFU of *Pichia kudriavzevii* (ASCUSDY21)/cow-daily is consistent other direct fed microbial products currently available to the cattle industry and published in the literature. The microbial **activity failure** by the *Pichia kudriavzevii* (ASCUSDY21) in performing expected improvement in nutrient (carbohydrate) ruminal degradation and generation of enzymes (phytases, proteases, and lipases) **should not impair the host ability to meet nutrients and energy requirements**. The dietary nutrient requirements for the expected level of production will be formulated independently from the presence of *Pichia kudriavzevii* (ASCUSDY21) or its activity. For instance, in case of complete failure in such additional microbial activity, the "inactive" microbes will simply be part of the pool of metabolizable protein delivered to the small intestine of the host. More specifically, the *Pichia kudriavzevii* (ASCUSDY21) has been offered to dairy cows at recommended dose in current dossier (noted by at least three publications included in the packet), in which animals did not show any adverse effect or signs of pathogenicity induced by the additional live microorganism included in the diet, other than positive effects on dairy cow's productivity.

The American Type Culture Collection lists ***Pichia kudriavzevii* as of little to no threat of infection in healthy humans and animals**. Literature search reported an outbreak of nine cases of opportunistic infection in neonatal at an intensive care unit due to *Pichia kudriavzevii*, which positively responded to voriconazole therapy. Mycotic mastitis is considered to be opportunistic and occurring in primarily immunosuppressed animals.

Moreover, *Pichia* species including *Pichia kudriavzevii*, are ubiquitous and have an established **history of use in the production of traditional fermented foods and beverages**, such as: wine; taruba (non-alcoholic cassava beverage); yakupa (spontaneously fermented non-alcoholic beverage consumed daily by children and adults); nunu (fermented yogurt-like milk beverage); gruel suanzhou (Chinese fermented cereal); and other Asian and African alcoholic beverages.

CONCLUSION AND FINAL CONSIDERATIONS

The scope of this report was to assess the safety, both target animal and human food products, of these direct fed microbial species used in ruminant diets. The approach used for the four microbial strains' isolation and identification from the rumen of healthy mid-lactation dairy cows is consistent with a safe rationale to use microorganisms intended to be used as direct-fed microbials. In addition to a safe approach rationale, current direct fed microbials were submitted to a: 1) unambiguous identification of strains using current phenotypic and genomic methods; 2) assessment of potential pathogenicity and virulence; 3) tests of stability and potential presence of contaminants; 4) test for potential antimicrobial resistance and mining whole genomes sequences for AMR genes to medically important antimicrobials; 5) peer-reviewed publication process where the commercial products were offered to lactating dairy cows; and 6) thorough analysis of current literature regarding any potential safety concern involving not only the specific strains, but also other members of the species and genus of each microorganism.

Therefore, based on the body of evidence submitted for current direct-fed microbial strains [*Butyrivibrio fibrisolvens* (ASCUSDY19); *Ruminococcus bovis* (ASCUSDY10); *Clostridium beijerinckii* (ASCUSDY20); and *Pichia kudriavzevii* (ASCUSDY21)], and comprehensive assessment performed by the expert panel, we do concur with the official GRAS conclusions (1st, 2nd, 3rd, and 4th) stated throughout the current report.

Prepared by:

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STUDY REPORT

A look at various conditions and blood types for evidence of hemolysin activity using a quantitative hemolysin assay

Contributors: (b) (6)

REPORT NO.	2024-001
REVISION NO.	00
REPORT DATE	12-MAR-2024

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STUDY REPORT

REPORT PREPARED BY		
(b) (6)	(b) (6)	3/12/2024
PRINT	SIGNATURE	DATE

STUDY REPORT APPROVALS

REPORT SIGNATURE APPROVALS		
Mallory Embree, PhD	(b) (6)	3/12/2024
CHIEF SCIENCE OFFICER	SIGNATURE	DATE
Kevin Korth	(b) (6)	3/12/2024
DIRECTOR OF REGULATORY AFFAIRS	SIGNATURE	DATE

PURPOSE

1. This study was performed in order to verify whether *Ruminococcus bovis* ASCUSDY10 exhibits hemolytic activity in a variety of stressed and non-stressed conditions. The quantitative hemolysin assay as described by Ridder *et al.*, 2021 is a method in which levels of blood cell lysis caused by microbial hemolytic exotoxins can be observed and measured. The assay was executed in this study to verify any notable levels of hemolysis.

EXPERIMENTAL METHODS

2. Protocol was adapted from Ridder *et al.*, 2021, with deviations as noted below.
3. Microorganisms tested in this study
   - **3.1.** *Staphylococcus hominis* ATCC 27844 (negative control not known to have hemolytic genes)
   - **3.2.** *Staphylococcus aureus* ATCC 25923 (positive control known to have hemolytic genes)
   - **3.3.** *Ruminococcus bovis* ASCUSDY10 (test strain)
4. Microbial culture conditions
   - **4.1.** Todd Hewitt Broth has been shown to promote production of hemolytic toxins in various *Streptococci* and *Staphylococci* species; therefore, this was the chosen media for this experiment (Baker *et al.*, 1973).
   - **4.2.** *Ruminococcus bovis* ASCUSDY10 would not grow without specific added components, so Todd Hewitt Broth medium #T47500 (b) (6), (b) (4) was amended with (b) g/L starch, (b) (4) g/L sodium acetate, (b) g/L yeast extract, and (b) (4) g/L cysteine hydrochloride.
5. (b) (4) mL of culture media was placed into (b) (4) tubes, sparged with (b) (4) N_2/(b) (4) CO_2 [v/v] for (b) (4) minutes, and then capped with butyl stopper and aluminum crimp seal prior to autoclaving at (b) (4) C. pH of media was checked post autoclave and adjusted in a sterile manner as necessary with either NaOH or HCl
6. Culture growth conditions were selected to mimic possible animal physiology stress conditions in order to induce potential hemolytic toxin production as shown in Table 1 below:

Medium pH	Incubation Temperature
pH 7.0	30°C
pH 7.0	37°C
pH 7.0	39°C
pH 5.5	37°C
pH 8.0	37°C

Table 1. Microbial culture pH and temperature growth test conditions

7. Peak hemolytic activity/toxin production occurs during late log phase/early stationary phase of bacterial growth (Beem *et al.*, 1998; Divyakolu *et al.*, 2019) so all cultures were grown to this point.

7.1. Both optical density measurements (OD_{600nm}) and Petroff-Hausser chamber microscope cell counts were completed on all cultures. (b) (4) cell counts were completed per the manufacturer's instructions (b) (6), (b) (4)

7.2. Blank media was added to *Staphylococcus aureus* cultures at end of growth to lower OD_{600nm} to 0.3 ± 0.02 in order to compare percent hemolysis between the three strains.

8. Cultures were processed as otherwise indicated in Ridder *et al.*, 2021.

9. Blood Products (purchased from (b) (6), (b) (4)

9.1. Sheep Blood in Alsevers ((b) (4))

9.2. Rabbit Blood in Alsevers (b) (4))

9.3. Ox Blood in Alsevers ((b) (4)

9.4. All blood was used within 21 days of draw date.

9.5. Blood was washed and processed as indicated in Ridder *et al.*, 2021

10. Microtiter Plate Preparation

10.1. Prepared samples (after normalization, centrifugation, and filtration as per RIdder *et al.* 2021) and blood were combined (b) (4) in a microtiter plate. Culture samples were substituted with 1% Triton-X 100 in 1X Phosphate Buffered Saline (PBS) for positive controls and with blank culture media as negative controls.

10.2. Plates were sealed and incubated at 37°C for (b) hours.

10.3. Plates were centrifuged at (b) (4) x g for (b) (4) mins and supernatant was transferred into a clean microtiter plate.

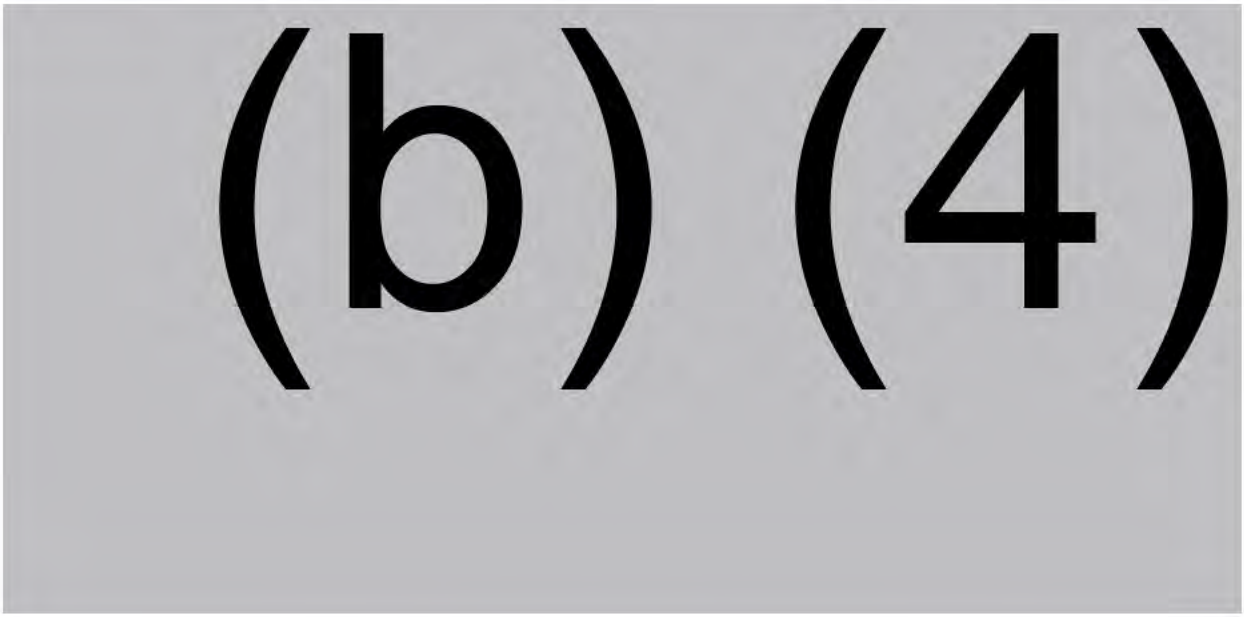


Figure 1. Supernatant of microbial culture/blood incubation transferred to clean microtiter plate.

10.4. Absorbance was measured at (b) (4) nm to detect any heme in order to calculate % hemolysis.

10.5. % Hemolysis calculation equation. All values were calculated using (b) (4) nm measurements (Beem et al., 1998; Walski et al., 2014)

$[(A_s - A_n) / (A_p - A_t)]*100$

A_s = sample absorbance

A_n = negative control absorbance (blank culture media/blood)

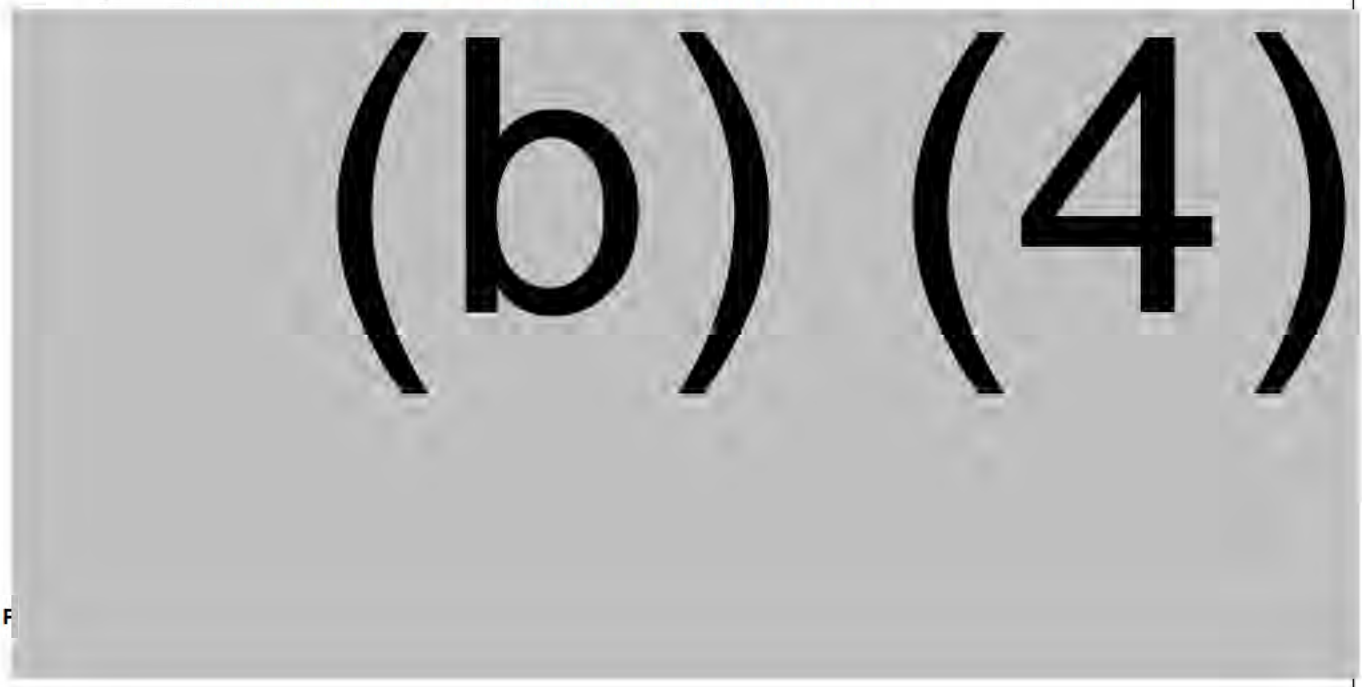
A_p = positive control absorbance

A_t = 1% Triton-X in 1X PBS only

10.6. All cultures were sampled at peak growth and single end sequenced (1x300bp) using an (b) (4). Sequencing results indicated no contamination of cultures.

RESULTS

11. Optical density 600 nm measurements for each culture were taken at the same time points for all fifteen cultures and tracked over time to determine microbial phase of growth. When it was determined based on O.D. $_{600nm}$ measurements that cultures would no longer continue to grow, they were normalized as necessary as instructed and described in the Ridder *et al.* 2021 protocol. (b) (4) cell counts were completed which showed cellular growth was comparable between strains in most growth conditions. O.D. $_{600nm}$ growth curve and (b) (4) chamber cell counts can be seen in Figure 2 and Table 2 below.



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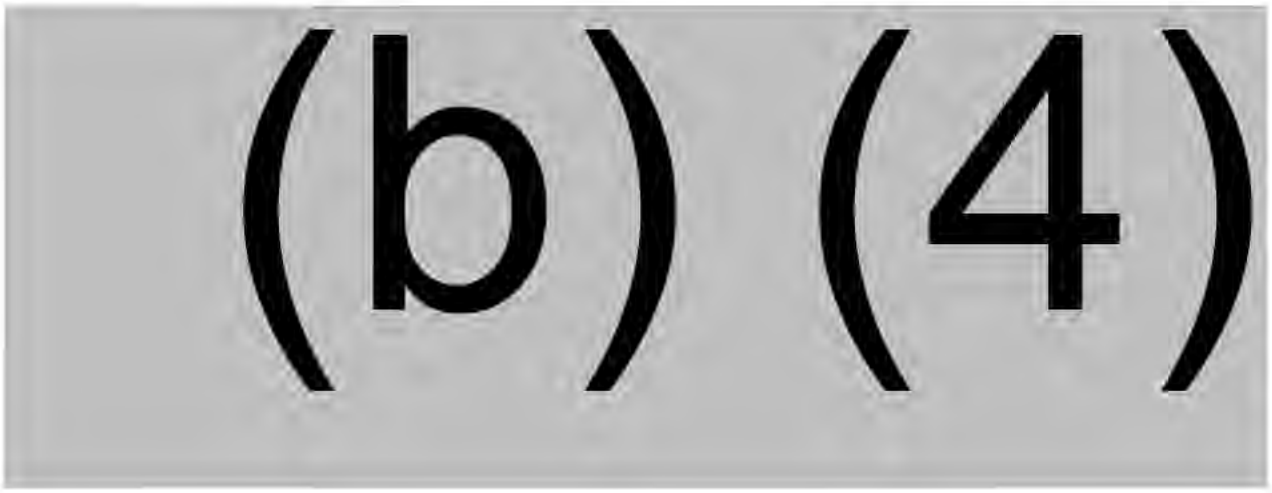

12. The calculated percent hemolysis of each culture separated by growth conditions and grouped by microorganism can be seen in both Figure 3 and Table 3 below. Out of the fifteen different conditions tested, DY10 showed very minimal hemolysis in only two of the conditions at 0.25% and 1.53%. *Staphylococcus hominis* (negative control microbe) exhibited hemolysis in three out of the fifteen conditions at 0.14%, 0.92%, and 3.42%. *Staphylococcus aureus* (positive control microbe) had notable hemolysis in 13 out of the 15 conditions tested ranging from 16.47-82.62% in the positive hemolysis conditions.

DY10 Quantitative Hemolysin Assay Results - Grouped by Microbe

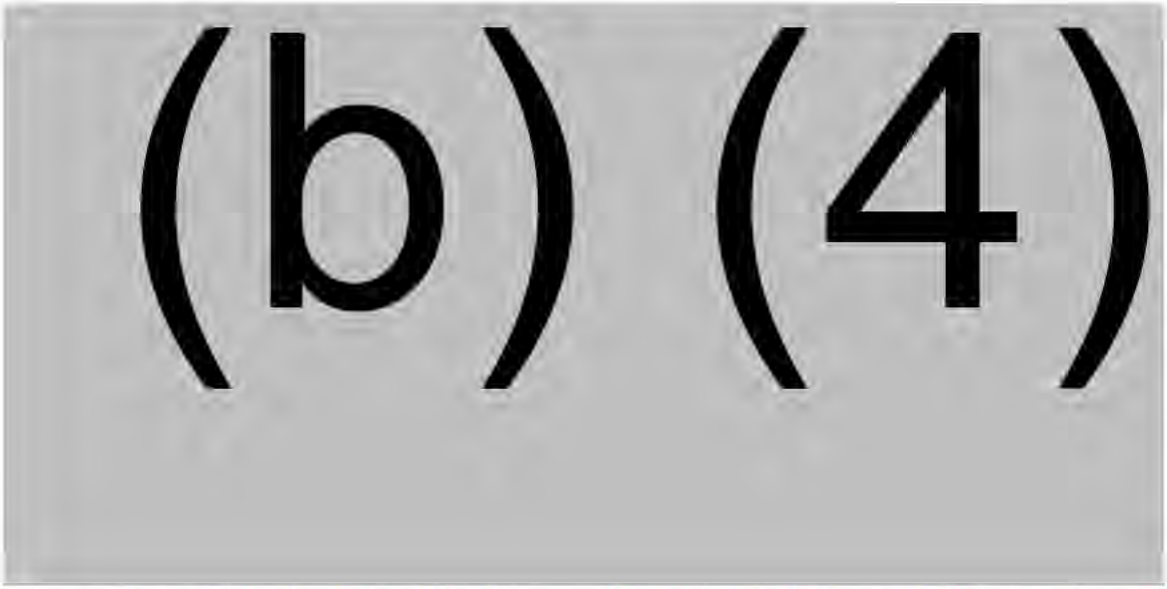

Figure 3. Calculated percent hemolysis of each culture and blood type. Percent hemolysis for each culture condition has been grouped be microorganism. DY is the test microbe DY10, SA is *Staphylococcus aureus*, and SH is *Staphylococcus hominis*.

Percent Hemolysis of All Conditions

Growth Condition	Microbe	Ox Blood % Hemolysis	Rabbit Blood % Hemolysis	Sheep Blood % Hemolysis
pH 5.5/37°C	SH	(b) (4)		
	DY			
	SA			
pH 7.0/37°C	SH			
	DY			
	SA			
pH 7.0/30°C	SH			
	DY			
	SA			
pH 7.0/39°C	SH			
	DY			
	SA			
pH 8.0/37°C	SH			
	DY			
	SA			

Table 3. Percent hemolysis of all tested culture conditions and animal blood types. DY is the test microbe DY10, SA is *Staphylococcus aureus*, and SH is *Staphylococcous hominis*.

13. (b) (6) sequencing results indicated no contamination present in any of the cultures

CONCLUSION

Ruminococcus bovis ASCUSDY10 exhibited 0.00% hemolysis in 13 different conditions as evidenced by the results of the quantitative hemolysin assay. While the two remaining conditions showed a hemolysis range from 0.25%-1.53%, the negative control microorganism (*Staphylococcus hominis*), a strain which does not contain any known genes for hemolysis, demonstrated a higher range from 0.14%-3.42% in three conditions. The positive control microbe (*Staphylococcus aureus*) displayed hemolytic activity in 13 of the 15 conditions: amounts which were higher than both the negative control and test strain microorganisms and ranged from 16.47-82.62%. Collectively, this data corroborates that DY10 does not exhibit hemolytic activity that would cause target animal safety or human safety concerns.

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September 10, 2024

David Edwards Director
Division of Animal Food Ingredients (HFV- 220)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

Subject: Amendment Animal GRAS Notice 68
DFM *Ruminococcus bovis ASCUSDY10* -
for Dairy Cattle

Notifier: Native Microbials, Inc.
1155 Island Avenue,
Suite 700
San Diego, CA 92101

Dear Dr. Edwards:

On behalf of Native Microbials, Iam providing an amendment to the GRAS Notice for the use of *Ruminococcus bovis ASCUSDY10* as a direct fed microorganism for use in Dairy Cattle. This is in response to the Division email of August 14, 2024 and August 26, 2024.

Please find the attached material in response to the issues raised by CVM. We consider our responses to be complete, and fully support the safety of *Ruminococcus bovis* ASCUSDY10

Should you have any questions on the filing, please contact me directly.

Sincerely,

Kristi O. Smedley, Ph.D.
Consultant to Native Microbials, Inc.

Cc: Kevin Korth, Native Microbials, Inc.

ATTACHMENTS:
Narrative response
2 Published References

AGRN # 68 *Ruminococcus Bovis* ASCUSDY10 GRAS Notice Amendment

The following represents the Native Microbials, Inc. response to the FDA-CVM questions in the email dated August 14, 2024 from Megan Hall, M.S to Kristi Smedley. The contents of the email are represented below, with the response below each question in blue text. Supporting documentation is contained in the referenced attachment.

Native Microbials, Inc. continues to conclude that *R. bovis* ASCUSDY10 is generally recognized as safe as a direct fed microbial in dairy cows at the intended rate of inclusion.

The statutory basis of GRAS is that there is a "reasonable certainty" in the minds of competent scientists that the substance is not harmful under the conditions of its intended use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance. Safety may be determined by scientific procedures or by general recognition of safety (21 CFR 570.30(a)). Native Microbials believes that it has met and exceeded the reasonable certainty of safety in its presentation of the notified substance.

List of Attachments:

1. Amended AGRN 68 Tables 2.20 and 2.21

Content of email from Animalfood-premarket@fda.hhs.gov and associated response

Genome Safety:

1. In the submission, the notifier indicates that *R. bovis* ASCUSDY10 sequence matched with 100% identity and 100% sequence coverage with the *R. bovis* (JE7A12T) (GCA_005601135.l) type strain sequence. We note that a total of 2,278 protein coding sequences of *R. bovis* ASCUSDY10 were predicted in the submission. However, predicted protein coding genes of *R. bovis* JE7A12T Type strain in NCBI are 2,220. The notifier should address this discrepancy.

   The *Ruminococcus bovis* ASCUSDY10 (JE7A12^{T}) genome was annotated using multiple platforms, each of which use different predictive algorithms and pipelines to determine the number of coding sequences. The protein coding genes were first annotated with the Rapid Annotation using Subsystem Technology (RAST), which uses GLIMMER2 and FIGfams as described in the dossier submission, and this resulted in the prediction of 2,278 coding sequences (CDSs). It was subsequently annotated with NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) as part of the resubmission & formalization of the nomenclature, which predicted there were a total of 2,243 coding sequences (43 of which were designated as pseudogenes/without protein) as indicated on the submission page of the *R. bovis* assembly (GCA_005601135.l). The NCBI annotation (2,200 CDSs) was used for all subsequent analyses presented in the

dossier, so the reported 2,278 CDSs was in error and referencing a previous genome annotation.

2. The notifier has not provided NCBI accession numbers for the identified BLASTp matches to potential toxin sequences in the *R. bovis* ASCUSDY10 genome listed in Table 2.21. Additionally, since the notifier has matched "ASCUSDY10 protein ID- Peg numbers" with databases, it is not clear which *R. bovis* ASCUSDY10 protein is queried against the databases. The notifier should provide NCBI accession numbers of each "Peg numbered" proteins. Without this critical information, CVM will not be able to evaluate notifier's safety conclusion.

   Thank you for bringing this to our attention. Tables 2.20 and 2.21 have both been amended to include the *R. bovis* ASCUSDY10 NCBI Accession numbers associated with the corresponding peg protein sequences. Please see amended Tables 2.20 and 2.21 in the attachment at the end of this response.

a. The notifier states that *R. bovis* protein, peg.1629, shares a similarity (28% identity, 59% coverage) to a hemolysin family protein (DBETH ID: D0HWk0) found in *Vibrio cholerae* in the Database for Bacterial ExoToxins (DBETH). The notifier further states that peg.1629 shares a higher identify (54% identity, 97% coverage) to a hemolysin family protein found in *Ruminococcus bromii*. The notifier tried to address the safety by arguing that *R. bromii* is not a known pathogen and no hemolytic activity has been reported in literature. This rationale is problematic as *R. bromii* has no history of safe use in food, nor has it been evaluated by CVM for safety in animal food. Referencing the 54% identity shared between *R. bovis* protein peg.1629 and the hemolysin family protein hit from *R. bromii* alone does not demonstrate the safety of peg.1629.

   We thank the CVM giving us the opportunity to provide more clarifying data. We feel it is important to point out that in our initial genomic interrogation (AGRN 53) at the cutoff of 70% identity and 70% coverage, none of the hemolysin genes were detected. Although FDA CVM has not publicly embraced the EFSA standard of 80% identity and 70% coverage (EFSA, 2021) for whole genome interrogation for toxigenic factors, no guidance has been provided by FDA CVM toward appropriate genomic cut-off values. The Pearson et al. 2013 values (30% similarity, 100% length, and E-value < 1E-06 to 1E-03, bits >50) presented in AGRN68 were provided to appease FDA CVM reluctance to accept EFSA standards, but the reality is the Pearson et al. cut-offs are more appropriate to measure homologies in context of evolution, e.g.

   > "*For analyses that depend on evolutionary distance, percent identity provides a useful approximation, but evolutionary distance is not linear with percent identity. The evolutionary distance associated with a 10% change in percent identity is much greater at longer distances. Thus, a change from 80% to 70% identity might reflect divergence 200 million years earlier in time, but the change from 30% to 20% might correspond to a billion year divergence time change.*"

   In hindsight, Native Microbials does not believe that the Pearson et al. standards are the most effective for detecting potential virulence and toxigenicity in modern microbial datasets, where the evolutionary timescale is much shorter and faster than the eukaryotes presented in the manuscript. **We concede that the basis of GRAS is the consensus of the scientific community, including the global scientific community, and would therefore include the scientists that sit on the panels that determine published EFSA requirements. Therefore, as there is no standard**

given by FDA, the EFSA standard, which represents the scientific community, becomes the standard and serves the requirements of GRAS. Only in response to CVMs request that we arbitrarily drop the cutoff threshold do we see any of these **partial matches**, which we then proceeded to address. These partial matches are no indication that a hemolysin active protein is produced and released by the organism.

This is exemplified by the approved AAFCO DFMs that contain the Hemolysin III gene above 30% Identity (as communicated in FDA meeting October 4, 2022):

Examples of Hemolysin III hits to AAFCO Organisms				
Organism	**Identity (%)**	**Coverage (%)**	**Annotation**	**AAFCO feature accession #**
Bacillus subtilis	71.3	93	hemolysin III	QJC89029
Bacillus amyloliquefaciens	69.8	93	hemolysin III	UFK55438
Lactobacillus helveticus	47.0	97	hemolysin III	QYH33668
Enterococcus faecium	45.8	97	hemolysin III	QKE87684

And additionally by the authorized AAFCO DFMs that contain the Hemolysin A (TlyA) gene above 30% Identity (as communicated in FDA meeting October 4, 2022)

Examples of Hemolysin A hits to AAFCO Organisms				
Organism	**Identity (%)**	**Coverage (%)**	**Annotation**	**AAFCO feature accession #**
Megasphaera elsdenii	46.8	98	TlyA family rRNA methyltransferase	AVQ74643
Streptococcus thermophilus	44.2	99	TlyA family RNA methyltransferase	AXT15581
Pediococcus damnosus	44.2	99	TlyA family RNA methyltransferase	AMV65775
Bacillus subtilis	44.1	99	TlyA family RNA methyltransferase	QVK13186

In all, as presented previously, hemolysin hits to various strains considered AAFCO approved above the 30% identity cutoff were found, including multiple strains of *M. elsdenii, S. thermophilus, S. intermedius, B. subtilis, B. licheniformis, L. helveticus, L. delbrueckii, L. acidophilus, E. lactis, E. faecium, P. damnosus, P. acidilactici, P. pentosaceus, P. animalis, S. thermophilus, S. intermedius, B. longum, B. adolescentis,* and *B. bifidum*. Despite this, however, none of these species are considered to have hemolysin activity, again supporting our understanding that the partial matches of individual genes alone are not indicative of a hemolysin-capable phenotype.

To address the question, Peg.1629 is less than 30% identity and 100% coverage proposed by Pearson et al 2013, so it should have not been included in the table. The amended Table 2.20 (attached) has removed all hits below the Pearson et al 2013 threshold.

b. The notifier states that *R. bovis* protein, peg.535, shares a similarity (51% identity, 94% coverage) to Hemolysin III (DBETH ID: Q897Y4) found in *Clostridium tentani* in the DBETH database. The notifier further states that peg.535 shares a higher identify (73.5% identity, 100% coverage) to a hemolysin family protein found in *R. bromii*. By citing a scientific article by Mahu et al. (2016), the notifier argues that in NCBI the domain of Hemolysin III is also found in proteins with functionally diverse, non-pathogenic membrane features. However, from reading the article by Mahu et al. (2016), CVM notes that despite the information provided above, the authors also point out "the most important genes involved in the strong hemolytic phenotype of *B.hyodysenteriae* are *tlyA*, *hlyA* and probably *hemolysin III*", and "Hemolysin III harbors a conservative domain yqfA, a predicted channel-forming protein of the Hemolsyin III family, which might indicate its role in *B.hyodysenteriae* hemolysis". Thus, CVM found that the literature information provided by the notifier is incomplete and contradicts its own safety conclusion regarding peg.535 and Hemolysin III.

Again, we point out that a 51% identity match means that nearly half the sequence is different and would not normally be of any particular concern and wouldn't have been notated had the cut-off matched thresholds defined by other regulatory bodies. Additionally, hemolysin family proteins are not necessarily hemolysins.

Regarding Mahu, et al., multiple strains of hemolytic and non-hemolytic strains of *B. hyodysenteriae* were compared. In the particular paragraph being cited, the full paragraph reads:

> The comparative sequence analysis of the hemolysis associated genes leads to a hypothesis with regard to the underlying mechanism of the weak hemolysis. The weakly hemolytic *B.hyodysenteriae* strain D28 possesses nucleotide sequence differences in the *tlyA*, *tlyB*, *hemolysin III*, *hemolysin activation protein* and *hemolysin III channel protein* genes resulting in amino acid substitutions. These sequences differ from those of all other strains in the study and from that of reference strain WA1. Whether the amino acid substitutions reported here are the sole reason for the weak hemolysis of this strain needs further studies. **In our opinion** the most important genes involved in the strong hemolytic phenotype of *B.hyodysenteriae* are *tlyA, hlyA* and probably *hemolysin III.* Deletion mutants for *tlyA* have been reported to be weakly hemolytic on blood containing agar plate [23]. The role of ACP in acylation of toxins has been demonstrated for other toxins, such as RTX toxins [24], which makes it likely that *hlyA* encoding an ACP plays a role in the hemolytic capacity of *B.hyodysenteriae.* Hemolysin III harbors a conservative domain yqfA, a predicted channel-forming protein of the hemolsyin III family, which **might** indicate its role in *B.hyodysenteriae* hemolysis. **Whether this reduced hemolytic capacity can be attributed to one of the amino acid changes in one of the hemolysis associated genes, remains to be determined.** In order to completely elucidate this, the construction of specific mutants of *B.hyodysenteriae* which harbor one of the divergent hemolysis associated genes is a prerequisite. This might be hampered by the fact that is difficult to genetically manipulate *B.hyodysenteriae.*

Our goal in citing this particular article is to present the presence of Hemolyin III in non-*Clostridium,* non-hemolytic microorganisms. In the manuscript, the authors show that weakly hemolytic / non-hemolytic members of *Brachyspira* harbor multiple mutations across multiple genes as compared to hemolytic members of *Brachyspira.* 5 genes (tlyA, tlyB, hemolysin III, hemolysin activation protein and hemolysin III

channel protein) are required for the strain to exhibit hemolytic activity, as identified through sequence mutations leading to amino acid substitutions. In context of *R. bovis* ASCUSDY10, only one of these genes was detected (hemolysin III at 51.2% identity and 94% coverage), suggesting that *R. bovis* ASCUSDY10 does not have the full genetic suite required to exhibit hemolytic activity. Furthermore, in the cited excerpt, the authors attempt to hypothesize the underlying mechanism for no hemolysis vs. strong hemolysis. It is worth noting that the importance of hemolysin III in conferring strong hemolytic activity is speculative based on their data and it has yet to be tested directly like tlyA and hlyA. Despite this, if we do accept that hemolysin III is a key gene involved in strong hemolytic activity, other genes are required to exhibit the phenotype which are not present in *R. bovis* ASCUSDY10. This was confirmed by our own *in vitro* testing (Appendix 021).

3. Regarding the *in vitro* hemolysis assay demonstrating non hemolytic activity by *R. bovis* ASCUSDY10, the notifier should explain the following:

   a. As indicated by the notifier, in the DBETH database the *R. bovis* protein, peg.535, shares highest similarity with Hemolysin III found in an obligate anaerobe, *Clostridium tetani* (causative agent of tetanus). Given that *R. bovis* is also an obligate anaerobe, it is reasonable to consider that Hemolysin III may require a strict anerobic environment to exhibit its normal function. Although the hemolysis assay presented in this notice was indeed carried out under anaerobic condition, the methodology and culture medium were adapted based on a protocol (Ridder et al., 2021) designed for a fast-growing and facultative anaerobic organism *Staphylococcus aureus* (i.e., can grow in both aerobic and anaerobic conditions). Since the positive control included was a *S. aureus* strain, it is unknown whether the adapted assay as presented is capable of correctly determining the potential hemolytic activity in obligate anaerobes such as *R. bovis*. For the proposed study, the notifier should have included a meaningful positive control, such as another obligate anaerobe which is known to produce hemolysis under the anaerobic condition. Otherwise, the notifier should use a method known to work for the determination of hemolytic activities in anaerobes. Therefore, CVM questions the suitability of the hemolysis assay used to demonstrate the non-hemolytic activity by *R. bovis*.

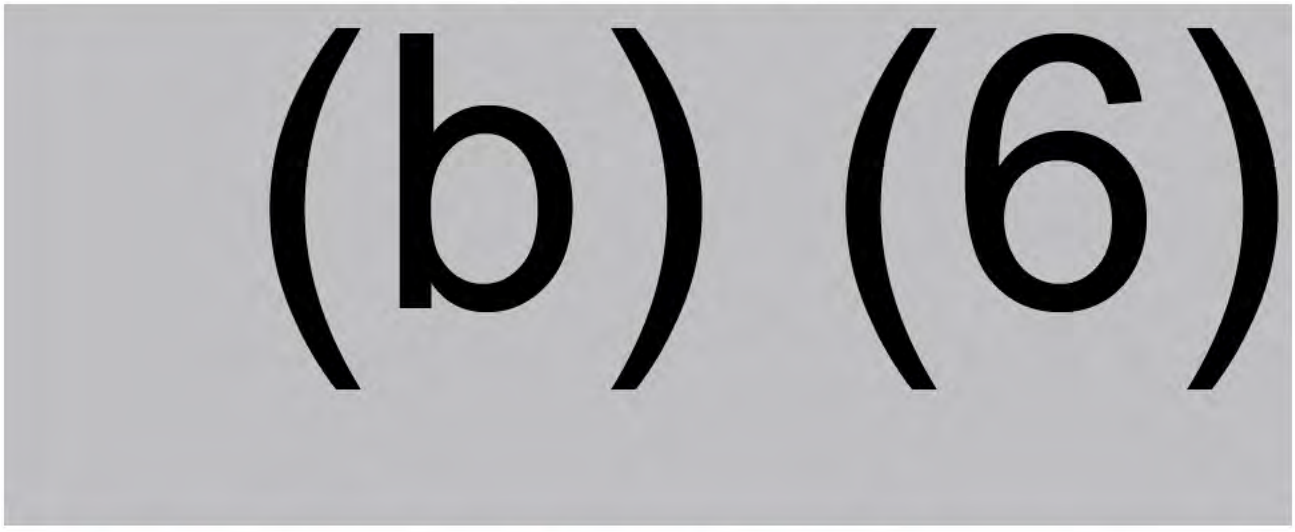
      As stated previously, had the notified substance been evaluated at the compendial 80% cutoff, 70% coverage, no hemolysin genes would have been interrogated from the genome of *R. bovis* ASCUSDY10. Only Hemolysin III was detected (at lowered identity and coverage cutoffs), and the other genes known to be required for a hemolytic phenotype were not detected at any threshold. As such, we do not believe there is a reasonable risk of hemolysin activity in the notified substance. Again, the hemolysin assay was done out of an abundance of caution and not because there was any suspicion or belief that there was any hemolysin capability in the notified substance.

      The quantitative hemolysin assay performed was adapted based on the protocol established by Ridder et al. 2021, which can be found in the book entitled *Staphylococcus aureus: Methods and Protocols (Methods in Molecular Biology, 2341)* by Kelly C. Rice. In this protocol, *Staphylococcus aureus* is the model organism and is a clear positively hemolytic microbe.

The strain of *Staphylococcus aureus* (ATCC 25923) chosen for our assay (described in Appendix 021) contains a hemolysin III protein (Accession YP_500893.1). When the hemolysin III protein sequence (peg.535/WP_138156423.1) of *R. bovis* ASCUSDY10 is BLASTed against that of *Staphylococcus aureus* strain, the two sequences show a 39.60% identity/93% query coverage. These values are within the same cutoff values (30% protein sequence identity with 70% query coverage) for protein alignments to toxin sequences used in Table 2.20 of the dossier. Therefore, *Staphylococcus aureus* is an adequate positive control microbe, as it contains an active hemolysin III suite and was also capable of growing under the same anaerobic & medium conditions as *R. bovis* ASCUSDY10.

Additionally, the quantitative hemolysin assay works with both facultative and obligately anaerobic bacteria. Native Microbials has completed the same quantitative assay using the obligate anaerobe *Clostridium perfringens* ATCC13124 as a positive control grown in unamended (b) (4) broth in addition to *Staphylococcus aureus*. *C. perfringens* also contains hemolysin III family proteins. Results from this assay were shared with FDA on October 4, 2022 in a discussion on AGRN 41 and AGRN 49 (note bar charts highlighting data presented in that discussion below). The obligate anaerobe *C. perfringens* positive control worked properly in that assay, right alongside of *S. aureus*, which was the same anaerobic assay setup as was performed for AGRN 68. Thus, there is no reason to believe the anaerobic assay and the clear hemolysis demonstrated by *S. aureus* presented in AGRN 68 was anything but properly controlled and effective in its execution.

Percent Hemolysis
May 2022 Quantitative Assay



b. CVM notes that the information on the NCBI website indicates that Hemolysin III family protein is an integral membrane protein that may have both hemolytic and cytotoxic activity against a broad range of species and cell types (https://www.ncbi.nlm.nih.gov/Structure/sparcle/archview.html?archid=10003362). The information provided in the DBETH database further indicates that the toxin

mechanism for Hemolysin III from *C. tetani* (match to peg.535 in *R. bovis* as presented in Table 2.20) is “Cytolysins” (http://www.hpppi.iicb.res.in/btox/cgi-bin2/new-introduction.cgi?name=Clostridium). Additionally, information from the literature suggests that some hemolysin-like toxin proteins do not possess hemolytic activities, but are cytotoxic for some cell types, e.g., the lethal Beta-toxin from *Clostridium perfringens* (DOI: 10.2217/fmb.09.72). For above reasons the evidence presented in the *in vitro* hemolysis assay, even properly designed, may still be insufficient to address the safety of the toxin proteins identified in *R. bovis* (e.g., Hemolysin III).

The cytotoxic effect of *Clostridium tetani* has been proven to be due to a tetanolysin, not Hemolysin III (Matsunaga et al). Further interrogation of *C. tetani* entries in DBETH show that none of the genes list “hemolysis” as a potential toxin mechanism. Listing “cytolysin” as a mechanism for “Hemolysin III” is likely a mis-annotation, especially since no primary literature was listed nor could we identify any primary literature linking Hemolysin III to cytotoxicity in *C. tetani.* The cytotoxic property of *C. tetani* is well established, with extensive research on the two exotoxins commonly produced (tetanolysin and tetanospasmin). Neither are present in *R. bovis.*

Considering that we do not reasonably believe there are active hemolysin genes and there is insufficient concern beyond a reasonable certainty of safety, the additional concern for cytotoxicity is moot. There was no indication in genomic interrogation that a *C. perfringens*-like beta toxin was present nor was there any evidence of *in vivo* activity of any cytotoxins in the nearly year-long feed trials that were done using the notified substance. We assert that this argument is a stretch beyond the reasonable certainty of safety, which is the criteria for GRAS (21 CFR 570(a)). Had this been a valid concern, the hemolysin genes discovered in genome interrogation for GRN 1090 *Bifidobacterium bifidum* strain NITE BP-31 a (strict anaerobe) viable microbe for use in human baby formula and approved by FDA CFSAN would have been refused for the same argument, which it was not.

Target Animal Safety:

1. CVM notes that *in vitro* models have limitations on how they can support target animal safety. The firm should adequately bridge *in vitro* data to the target animal, by providing additional narrative regarding how results and conclusions from *in vitro* studies correlate to the safety of the *R. bovis* strain when fed to dairy cattle.
    a. *[additional clarification per the email received 8/26/24 from Megan Hall at Animalfood-premarket@fda.hhs.gov]* The CVM comment you question is referring to in vitro hemolysis assays. Our concerns are two-fold.
    b. First - with respect to the firm’s approach to use an in vitro hemolysis assay to explain their conclusion that hemolysins in *R. bovis* do not constitute a TAS risk – the assays, as described, may not be adequately designed, or controlled.
    c. Second - published literature suggests hemolysins (the proteins of concern) can cause cytotoxicity independently of observed hemolysis (the act of rupturing red blood cells from the assay). The hemolysis assay does not address the hemolysis-independent cytotoxicity of the proteins of concern (hemolysins) nor does the assay address all the stressors that may be encountered in the rumen of the target animal. Additionally, the assay only evaluates hemolysin production and possible activity at different temperatures and pHs. The assay does not evaluate other stressors that may trigger hemolysin production in the

rumen of the target animal. Literature suggests that factors other than pH and temperature may induce hemolysin production by microorganisms, including microenvironment, available nutrients/metabolites, neighboring microbes, etc. Therefore, the in vitro hemolysis assay alone is not sufficient to address the potential impact on TAS, and characterizing the identified hemolysins and their potential pathogenesis using published literature may help address the target animal safety concerns

Native Microbials has adequately explained in the prior questions that the hemolysin assay was adequately controlled and the design was consistent with current published literature. We have also explained that the gene itself was a poor match, well below thresholds recognized by the scientific community as needing further investigation and as explained previously, the cytotoxic inference is baseless considering the required genes are not present. There is no literature linking the hemolysin III gene to cytotoxicity.

Native Microbials had numerous discussions with FDA CVM on the topic of when additional target animal safety studies, beyond published long-term feeding studies are warranted with viable microbes. We understand that conditions exist where high homology matches to potentially toxigenic genes or genera of historical concern drive up that additional need, we do not believe that threshold has been met with *R. bovis* ASCUSDY10, nor do we believe it should always be done regardless. The *in silico* genetic potential for toxicity or pathogenicity has been exhaustively demonstrated to be extremely low and group II *Ruminococcus* are well known to be non-pathogenic, highly prevalent animal microbiome commensals in the literature. As such, we do not agree that additional studies are needed to bridge the presented *in vitro* data with this organism.

As such, we believe we have met the ***reasonable*** certainty of safety required for GRAS (§ 570.30(a))

2. For assessment of target animal safety, the experimental designs of the submitted *in-vivo* studies are cofounded because the variable being evaluated, *R. bovis*, was not evaluated in isolation, but rather administered with other viable microbes that are not approved or otherwise acceptable for use in animal food in the United States. Because viable microorganisms are expected to reproduce and grow, their interactions can impact the quality and accuracy of the data concerning one of the organisms in a consortium. In other words, it is unclear if the lack of any noted/recorded adverse events are consequent to animals being tolerant to the notified substance, or if apparent tolerance is a result of suppressive interaction(s) from different microbial species when fed concomitantly. Do you have any studies where *R. bovis* has been administered as the sole test article?

   Native Microbials continues to assert that the data provided meets the criteria for a reasonable certainty of safety. In context, viable microbes are naturally present and consumed by dairy cattle regardless of whether additional microorganisms are administered. The consortia of microorganisms used in the studies are commensal organisms commonly found in healthy, high-producing dairy cattle. Administering a single microorganism does not negate the fact that these and other microorganisms are already naturally present in the rumen and gastrointestinal tract (GIT).

It is impossible to administer a single commensal microorganism in isolation due to the presence of millions of microorganisms that could influence its function. For example, silage contains uncharacterized organisms at concentrations far higher than those involved in the administration of the notified substance. Similarly, naturally present rumen organisms often occur at much higher concentrations than the intended rate of inclusion due to the sheer size and volume of the rumen.

Therefore, we assert that administering the notified substance alongside three other naturally occurring rumen organisms should not invalidate the long-term feeding studies presented. If the substance were harmful to dairy cattle, this would have been evident in the extensive studies—270 days in Valldecabres et al. (2022), 112 days in Goldsmith et al. (2023), and 143 days in Dickerson et al. (2022)—all of which were published in peer-reviewed journals. Thus, a reasonable certainty of safety *in vivo* has been demonstrated not once but three times through published, peer-reviewed scientific research.

Had any negative effects arisen from feeding the consortia product, it would have been challenging to isolate which of the four organisms caused the issue. However, as all studies reported no negative findings, we can reasonably conclude that the notified substance poses no toxigenic or pathogenic risk to the target animal.

Although there are no studies where *R. bovis* was administered as the sole test article to lactating dairy cows, its role as a core member of the rumen microbiome in dairy cows was thoroughly demonstrated in AGRN 68. Thus, *R. bovis* will always be present in the rumen at varying levels, even without supplementation. Given today's technology, it is impossible to create germ-free ruminants, making it infeasible to test the impact of *R. bovis* in an environment devoid of naturally occurring populations.

Finally, it is highly unlikely that any suppressive interactions occur when feeding *R. bovis* as part of a consortia. The other microbial species used in the animal studies were also sourced from the core rumen microbiome, meaning all microbes administered are naturally present in the rumen across most cows. Even if *R. bovis* was fed in isolation, we would expect the same absence of adverse effects since the other microbes in the studies are naturally present in the rumen microbiome.

Prior submissions by Native Microbials have shown that the other three organisms used in the feed studies are naturally present in the rumen: *P. kudriavzevii* (8-20%, AGRN 38), *C. beijerinckii* (0.2-3%, AGRN 41), and *B. fibrisolvens* (1E-03 to 1%, AGRN 42). The assertion that interference could occur goes beyond the reasonable certainty of safety. Such an argument could apply to any substance, feed additive, or drug submitted for FDA review, as there will always be other organisms present in the rumen and feed of dairy cattle that could potentially cause suppressive interactions.

ADDITIONAL REFERENCES CITED

1. EFSA. 2021. EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. EFSA Journal doi: 10.2903/j.efsa.2021.6506.
2. MATSUNAGA, T., MIYAMOTO, K. and KOSHIURA, R., 1982. Cytotoxic effect of the culture supernatant of Clostridium tetani. *Chemical and Pharmaceutical Bulletin*, *30*(2), pp.702-707.

ATTACHMENT 1: AMENDED AGRN 68 TABLES 2.20 AND 2.21

Amended Table 2.20: *R. bovis* ASCUSDY10 Significant Protein Alignments to DBETH Toxin Sequences

ASCUSDY10 protein ID	DBETH ID	ASCUSDY10 NCBI protein Accession #	DBETH Annotation	Source Organism	Subject Coverage	Query Coverage	% Identity	E-Value
peg.535	Q897Y4	WP_138156423.1	Hemolysin III	*Clostridium tetani*	96	94	51.2	2.00E-66
peg.1931	Q73VP2	WP_175405453.1	LepB	*Mycobacterium paratuberculosis*	31	35	33.3	1.00E-05

Amended Table 2.21: BLASTp Matches in NCBI to Potential Toxin Sequences in the *R. bovis* ASCUSDY10 Genome

ASCUSDY10 protein ID	ASCUSDY10 NCBI protein Accession #	Organisms providing best match by BLAST	Annotation of closest related protein in NCBI	Identity (%)	Query Coverage (%)
peg.529	WP_138156419.1	*Paenibacillus crassostreae*	ABC transporter ATP-binding protein	64.8	100
peg.1416	WP_138157207.1	*Ruminococcus bromii*	lectin like domain-containing protein	36.6	97
peg.204	WP_138156133.1	*Ruminococcus bromii*	beta-ketoacyl-ACP synthase II	75.4	99
peg.530	WP_138156420.1	*Sporobacter termitidis*	ABC transporter permease	48.8	99
peg.891	WP_138156726.1	*Caproiciproducens galactitolivorans*	hemolysin family protein, HlyC/CorC family transporter	55.5	92
peg.731	WP_138156589.1	*Ruminococcus bromii*	Predicted Zn-dependent peptidase	50.8	99
peg.2055	WP_138157746.1	*Roseburia hominis*	ABC transporter ATP-binding protein	68.8	96
peg.216	WP_175405327.1	*Ruminococcus bromii*	MATE family efflux transporter	66.4	100
peg.1184	WP_138157020.1	*Clostridium porci*	4'-phosphopantetheinyl transferase superfamily protein	33.5	89
peg.969	WP_138156791.1	*Acetivibrio straminisolvens*	AMP-binding protein	43.5	97
peg.1430	WP_138157218.1	*Ruminococcus bromii*	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A	78.4	100
peg.2072	WP_138157760.1	*Ruminococcus bromii*	ABC transporter ATP-binding protein	88.9	100

Table 2.21 (cont.): BLASTp Matches in NCBI to Potential Toxin Sequences in the *R. bovis* ASCUSDY10 Genome

ASCUSDY10 protein ID	ASCUSDY10 NCBI protein Accession #	Organisms providing best match by BLAST	Annotation of closest related protein in NCBI	Identity (%)	Query Coverage (%)
peg.402	WP_138156307.1	*Allobaculum stercoricanis*	cardiolipin synthase	57.0	100
peg.312	WP_138156231.1	*Eubacterium ruminantium*	putative transcriptional regulator	85.7	98
peg.541	WP_138156427.1	*Mediterraneibacter* (*Ruminococcus*) *gnavus*	S8 family serine peptidase	31.7	99
peg.1128	WP_138156972.1	*Blautia glucerasea*	patatin family protein	43.4	99
peg.709	WP_138156569.1	*Clostridium innocuum*	DUF5963 family protein	55.9	100
peg.2056	WP_138157747.1	*Longicatena caecimuris*	Ig-like domain-containing protein	32.7	58
peg.535	WP_138156423.1	*Ruminococcus bromii*	hemolysin III family protein	73.5	100
peg.1629	WP_022505557.1	*Ruminococcus bromii*	hemolysin family protein	54.4	97
peg.1931	WP_175405453.1	*Sharpea porci*	signal peptidase I	54.3	68
peg.1732	WP_138157466.1	*Ruminococcus bromii*	pitrilysin family protein	62.9	100
peg.1356	WP_138157179.1	*Ruminococcus bromii*	UDP-N-acetylglucosamine pyrophosphorylase	78.6	99
peg.2251	WP_138157903.1	*Ruminococcus bromii*	serine/threonine protein kinase	57.6	94